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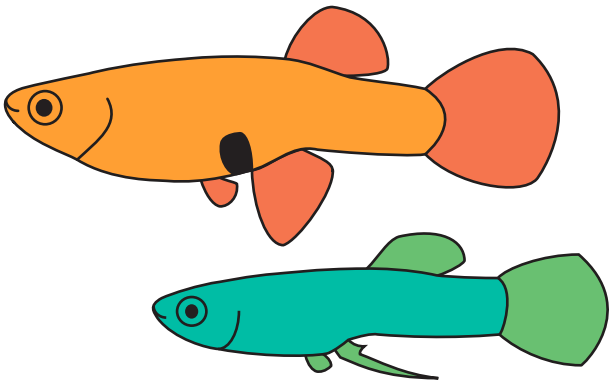
Inbreeding depression and a poor start in life



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Declaration

The research presented in this thesis is my own original work. All of the chapters are co-authored. The authorship order indicates the intellectual input and workload. No part of this thesis has been submitted for any previous degree.

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Abstract

Inbreeding is a widespread phenomenon that can decrease fitness. Inbreeding depression occurs because matings between relatives lead to an increase in homozygosity. Inbreeding is a pervasive force in evolutionary ecology driving the evolution of different traits, mating systems, and influencing population dynamics. It is generally assumed that the negative effects of inbreeding are exacerbated in stressful environments. In this thesis, I present seven experimental studies that explore whether life history, morphological, and sexual traits show inbreeding depression, and if this effect is increased by an interaction with an early stressful environment.

Chapter 1 explores the preference for novel mates by males and females depending on the choosers' previous sexual experience. I discuss the potential adaptive significance of these preferences and the likelihood of there being benefits of mating with multiple partners for both males and females.

In the second chapter I look at the effects of mating with relatives on offspring fitness. I highlight the importance of considering the potential role of maternal effects when studying inbreeding depression, and the relative importance of genetic and maternal effects on reproductive traits and offspring performance.

Chapter 3 addresses the interaction between inbreeding depression and an environmental stress, in the form of restricted food availability early in life. I test whether diet restriction during early development influences subsequent growth trajectories in ways that depend on the level of inbreeding. I then discuss potential hidden long-term costs that could affect reproductive success.

In the fourth chapter I investigate the effects of limited food availability on sexually selected traits. I present a study testing whether a poor early diet is costly due to the reduced expression of sexually selected male characters. I aim to understand whether individuals are able to compensate for a poor start in life in various ways, or if they still incur costs that are evident after maturation.

Chapter 5 investigates how differences in inbreeding and an early stressful environment influence the actual reproductive success of males. I argue about the extent to which inbreeding depression in males is due to natural or sexual selection.

In Chapter 6 I explore how key factors act and interact to determine the strength of parental effects and whether these factors differ between mothers and fathers, and between their effects on sons and daughters. I discuss the multifaceted role of parental effects in a species lacking parental care.

Finally, in the seventh chapter I provide a test of the effects of early life environment on the expression of genetic and maternal effects variance for a range of adult traits. I argue that maternal by-environment interactions are an under-appreciated component of phenotypic diversity.

Thesis outline

The following chapters compose this thesis:

1. The effects of familiarity and mating experience on mate choice in mosquitofish, *Gambusia holbrooki*
Behavioral Ecology 2014 25 (5): 1205-1211

2. Evidence for inbreeding depression in a species with limited opportunity for maternal effects
Ecology and Evolution 2015 5 (7): 1398-1404

3. Inbreeding depression does not increase after exposure to a stressful environment: a test using compensatory growth
BMC Evolutionary Biology 2016 16 (1): 68

4. Are sexually selected traits affected by a poor environment early in life?
BMC Evolutionary Biology 2016 16 (1): 263

5. Experimental evidence for sexual selection against inbred males
Journal of Animal Ecology 2017 86 (2): 394-404

6. What happens to offspring when parents are inbred, old or have had a poor start in life?
Accepted in *Journal of Evolutionary Biology*

7. Maternal-environment but not genotype-environment interactions in a fish without parental care
Heredity 2018 120 (2): 154-167

Appendix 1. Why does inbreeding reduce male paternity? Effects on sexually selected traits

Evolution 2017 71 (11): 2728-2737

Appendix 2. Testing female preferences under more natural conditions: a case study on a fiddler crab

Behavioral Ecology and Sociobiology 2017 71 (5): 81

Appendix 3. Predictors of male insemination success in the mosquitofish (*Gambusia holbrooki*)

Ecology and Evolution 2015 5 (21): 4999-5006

Appendix 4. Maternal effects on offspring size and number in mosquitofish, *Gambusia holbrooki*

Ecology and Evolution 2015 5 (14): 2945-2955

Appendix 5. Male mate choice and insemination success under simultaneous versus sequential choice conditions

Animal Behaviour 2015 103: 99-105

Contents

Introduction

Inbreeding and inbreeding depression

Inbreeding is one of the most important topics in evolutionary biology and conservation genetics. Inbreeding is common in small, fragmented populations, where mating among relatives occur (Keller and Waller 2002; Becker et al. 2016). However, inbreeding is ubiquitous and occurs not only in small but also large populations, making it a universal phenomenon, which has been documented in many different organisms (Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Keller and Waller 2002; O'Grady et al. 2006; Charlesworth and Willis 2009). Inbreeding refers to the mating of closely related individuals that are genetically similar, and inbreeding depression refers to the reduction in fitness of offspring of these matings, compared to the offspring of randomly mated individuals (Hedrick and Kalinowski 2000).

Inbreeding depression is known to occur due to an increase in homozygosity. That is, recessive or partially deleterious alleles, which occur usually at low frequency and are relatively unexposed to selection, have an increased probability of being homozygous and thus expressed in inbred individuals (Charlesworth and Charlesworth 1987; Keller and Waller 2002; Charlesworth and Willis 2009). Alternatively, and probably less common, inbreeding depression occurs due to loci that show a heterozygous advantage (i.e. overdominance) that are more likely to be homozygous in inbred individuals (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009; Pemberton et al. 2016).

The consequences of an increase in homozygosity are often negative. This is why inbreeding tends to lower the values of fitness related traits and is usually detectable as lower fertility, survival, and growth rates, as well as reduced resistance to predation, disease, and environmental stress (Keller and Waller 2002; Roff 2002; Charlesworth and Willis 2009). Given the detrimental effects on fitness, inbreeding depression can critically influence the evolution of mating systems (Byers and Waller 1999; Armbruster and Reed

2005). For instance, inbreeding can shape mate choice and select for the avoidance of related individuals to prevent the production of inbred offspring and/or through the avoidance of low-quality individuals, who are often inbred (Frommen et al. 2008; Ilmonen et al. 2009; Pilakouta and Smiseth 2017).

Inbreeding depression and inbreeding avoidance

It is commonly assumed that inbreeding depression is a selective force for the evolution of reproductive strategies that result in inbreeding avoidance mechanisms (Pusey and Wolf 1996; Szulkin et al. 2013). These strategies include sex-biased dispersal, specific choice of unrelated mates, mechanisms of kin recognition, and kin-based sperm selection (Pusey and Wolf 1996; Bretman et al. 2004; Szulkin et al. 2013). For example, females can bias fertilization towards sperm from genetically compatible males (Tregenza and Wedell 2002). However, the fitness costs associated with avoiding inbreeding can lead to a lack of inbreeding avoidance in some cases (e.g. Jennions et al. 2004; Tan et al. 2012), or even an apparent preference for inbreeding (Kokko and Ots 2006; Robinson et al. 2012).

Selection to avoid inbreeding can influence a range of behavioural traits (Charpentier et al. 2007). For instance, individuals often avoid mating with relatives that they encounter as potential mates, which implies the ability to discriminate kin from non-kin (Pusey and Wolf 1996; Charpentier et al. 2007). Similarly, genetic relatedness can influence fertilization success through post-copulatory mechanisms (Bretman et al. 2004; Evans et al. 2008). The probability of genetic incompatibility can also decrease when individuals mate with multiple partners (Gershman 2009). Mating preferences can thus lead to discrimination against previous mates, which has been shown in a wide array of taxa (Zeh et al. 1998; Archer and Elgar 1999; Eakley and Houde 2004), although this is not always the case (e.g. Fromhage and Schneider 2005; Gershman and Sakaluk 2009).

Mating preferences can lead to a preference for novel partners over previous mates, which can increase the genetic benefits for their offspring (Jennions and Petrie 2000; Mays and Hill 2004). In some cases, discrimination against familiar mates can result in increased fertilization success for novel mates (Gershman and Sakaluk 2009). An

increased mating effort invested into obtaining novel mates is known as the 'Coolidge effect', which refers to the progressive decline in mating with a previous partner, but a renewed sexual interest when a novel partner is available (Dewsbury 1981). This ability to discriminate familiar individuals could lead to discriminate against related individuals.

The benefits of choosing novel mates might be important to avoid mating with genetically incompatible mates, or to ameliorate the negative effects of mating with relatives (Stockley 1999; Tregenza and Wedell 2000, 2002). While the Coolidge effect is not the only way to avoid inbreeding, it is a mechanism by which inbreeding can be avoided. However, the evolution of these behaviours depends on the individual's plasticity in response to the costs associated with inbreeding depression (Charpentier et al. 2007). Additionally, inbreeding avoidance can depend on the selection pressures experienced in particular systems, such as the risk of inbreeding and variation in tolerance to inbreeding (Kokko and Ots 2006).

Inbreeding depression and its effect on different traits

Different genotypes carry different alleles that lower the fitness of homozygotes, causing great variation from one genotype to another (Charlesworth and Charlesworth 1999). Thus, inbreeding can negatively influence phenotypic traits that are associated with fitness and shape many life history traits across species and environments (Charpentier et al. 2007). Life-history traits are expected to be more strongly affected by inbreeding depression than morphometric traits given their higher level of dominance variance (Roff 1997; DeRose and Roff 1999; Coltman and Slate 2003), perhaps reflecting stronger directional selection for fitness-related traits (Falconer and Mackay 1996). Sexual traits are traits that can also be affected by inbreeding depression by either changes in heterozygosity at loci that directly code for sexual traits (Valtonen et al. 2014), or due to capture of genetic variation in condition (Rowe and Houle 1996; Prokop et al. 2010). Additionally, the extent of inbreeding depression can vary between males and females (Ebel and Phillips 2016).

The magnitude of inbreeding depression can vary with the fitness trait or life history stage measured (Angeloni et al. 2011). Inbreeding depression may not be detected

when only a few components of fitness are examined. For example, lack of inbreeding depression for juvenile survival does not mean there is no inbreeding depression for fecundity or mating success, or that different fitness components do not interact to reduce overall fitness (Hedrick and Kalinowski 2000; Keller and Waller 2002). Studies should examine multiple fitness traits across several life stages to look at how inbreeding depression acts early and late in life. Additionally, although inbreeding appears to reduce fitness, its magnitude and specific effects are highly variable and can depend on the genetic constitution of species or populations, and how these genotypes interact with the environment (Hedrick and Kalinowski 2000).

Inbreeding depression and the environment

The fitness of an individual is a function of its intrinsic quality, which includes the expression of any deleterious alleles, and extrinsic factors, such as the environment an individual is exposed to (Pemberton et al. 2016). This often leads to inbreeding depression only being detected under certain environmental conditions. That is, the effects of inbreeding depression are usually more readily detectable under competition (Meagher et al. 2000; Michalczyk et al. 2011; Simmons 2011) and/or environmental stress (Armbruster and Reed 2005; Fox and Reed 2011).

There is evidence in both plants and animals of interactions between inbreeding depression and the environment. Inbred individuals are more sensitive to environmental stress, presumably because stress increases the expression of deleterious recessive alleles (Fox and Reed 2011; Reed et al. 2012). Alternatively, it can be the result of genotype-by-environment interactions, which arise through condition-dependent deleterious alleles that are neutral or beneficial in benign environments, but deleterious in stressful environments (e.g. Bijlsma et al. 1999; Kristensen et al. 2003). A meta-analysis on inbreeding depression and stressful environments showed an increase of inbreeding depression in more stressful environments (Armbruster and Reed 2005). However, only 48% of the studies showed a statistically significant increase. Additionally, it has been suggested that the effect of the environment on inbreeding depression scales linearly with the magnitude of stress (Fox and Reed 2011). It is still unclear how stressful environments impact the relationship between inbreeding depression and fitness. Inbreeding usually

reduces mean fitness relative to outbred individuals, but there is conflicting evidence on whether it is consistently exaggerated in stressful environments (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000). This suggests that the intensity of the interaction between inbreeding depression and the environment depends on the study population and the type of stress that individuals are exposed to (Reed et al. 2012).

An example of a stressful environment — compensatory growth

The environment that individuals experience early in life has a substantial influence on their phenotype and, in turn, on their reproduction and survival (Bize et al. 2006). Resource availability during early periods of life can strongly affect an individual's fitness (Auer 2010; Dmitriew 2011). For instance, periods of poor resource availability can delay maturation and result in individuals having a small body size at maturation leading to lower adult survival and reduced reproductive success (Roff 1992; Stearns 1992). Given the potential fitness costs of a small body size, selection might favour mechanisms that mitigate the negative effects of small size early in life (Metcalfe and Monaghan 2001).

One of the main environmental factors that shape growth and reproductive success is food availability (Festa-Bianchet et al. 2000; Bize et al. 2006). Food availability determines how individuals allocate resources towards growth, immunity, or maturation (Metcalfe and Monaghan 2001; Taborsky 2006). Poor early nutrition has been shown to have negative effects on body size, survival, and secondary sexual traits (Lindström 1999; Metcalfe and Monaghan 2001)). Thus, low food availability early in life can have negative consequences for fitness (Auer 2010). However, if nutritional conditions improve, individuals can compensate for the negative impact of poor nutrition on growth (Metcalfe and Monaghan 2001). Individuals can either accelerate their growth rates (catch-up growth) and/or take longer to reach maturity (compensatory growth) (Metcalfe and Monaghan 2001; Ali et al. 2003; Hector and Nakagawa 2012).

The potential benefits of compensatory and catch-up growth can be outweighed by long-term costs on sexually selected and life history traits (Royle et al. 2005; Auer 2010; Kahn et al. 2012; Lee et al. 2012). A greater relative investment in somatic maintenance might increase the potential breeding lifespan of an individual, but reduce its likelihood

of obtaining a mate due to reduced investment in ornaments or body size (Lindström et al. 2005). There is potentially a trade-off between compensating early in life and impairing fitness later in life. Interestingly, the costs associated with a poor nutrition early in life might not be evident until later in life. This can result in adults that are superficially identical despite having experienced different environmental histories. For example, zebra finches experiencing low quality nutrition during the nestling period are morphologically similar to their counterparts, but have a lower capacity to assimilate antioxidants, which could affect their ageing rate (Blount et al. 2003).

Dealing with early stressful environments — a special case: sperm traits

Individuals that have experienced a stressful environment early in life often allocate more energy to somatic maintenance (Runagall-McNaull et al. 2015). However, developmental diet can affect traits expressed later in life and differentially affect investment into naturally and sexually selected traits. Sexual traits are typically strongly condition-dependent (Cotton et al. 2004) and early life condition can influence their expression (e.g. Sentinella et al. 2013; Fricke et al. 2015). Pre-copulatory sexually selected traits can be detrimentally affected by poor early nutrition. For example, pheasants given high quality food during the first weeks post-hatching are redder at sexual maturity, which increases their sexual attractiveness (Ohlsson et al. 2002). Given lower food resource availability, males might also invest differently in traits under pre-copulatory and post-copulatory sexual selection (e.g. Devigili et al. 2013; Rahman et al. 2013).

Male reproductive success does not solely depend on the ability to compete for matings (pre-copulatory traits), but also on the ability to fertilize females (Bretman et al. 2014), particularly when sperm competition is intense. Therefore, males also have to invest in sperm traits, which are costly to produce (Bunning et al. 2015). Sperm performance depends on sperm quantity (ejaculate size and percentage of motile sperm) and quality (sperm swimming velocity and longevity (Snook 2005; Pizzari et al. 2008; Birkhead et al. 2009; Parker and Pizzari 2010). Sperm production is condition-dependent, but whether early diet affects these traits is less well known (but see Tigreros 2013; Cordes et al. 2015). The potential of early diet to influence sexual traits reflects the importance of

looking at how past experiences shape adult traits.

Effects on the next generation — parental effects

Parents affect how their offspring look and behave. Most obviously, offspring resemble their parents because of the genes they inherit. However, the direct effects of inherited genes are not the only factors that determine offspring's phenotype (Youngson and Whitelaw 2008; Bonduriansky and Day 2009). Parental effects describe any situation in which parent's phenotype can affect their offspring phenotype (Räsänen and Kruuk 2007; Wolf and Wade 2009). That is, non-genetic inheritance such as effects of parental genotype, phenotype, and environment can influence offspring fitness (Mousseau and Fox 1998; Fay et al. 2016). For example, the prenatal environment a mother experiences can later affect her offspring's phenotype through its effects on her investment in egg development or yolk deposition (e.g. Hubbard et al. 2015; Merkling et al. 2016).

Environmental conditions experienced by parents such as nutritional level, toxin exposure, and stress can affect their offsprings' fitness (Mousseau and Fox 1998; Uller 2008; Burton and Metcalfe 2014). Similarly, parent's conditions such as their inbreeding status or state of senescence could influence their offspring's phenotype (e.g. Mattey et al. 2013; Schroeder et al. 2015). Inbreeding is known to have multigenerational effects on fitness, but it is unclear whether inbreeding-stress interactions persist across generations (Reed et al. 2012). There is, however, increasing evidence for indirect costs of inbreeding due to reduced parental investment by inbred parents. For example, studies on birds have shown that offspring of inbred parents have a lower immune response (Reid et al. 2003), lower fledging, and lower reproductive success (Szulkin et al. 2007), and may show lower incubation attentiveness and lower hatching success compared to offspring from outbred parents (Pooley et al. 2014). Even so, there is limited understanding of the potential interplay between the multiple causes of transgenerational effects, and whether early parental effects influence later life history stages of their offspring.

Heritability

Evolutionary responses require traits to have a heritable basis (which is usually synonymous with additive genetic variation, the quantification of which is essential for understanding the causes of phenotypic variation in traits (Mousseau and Fox 1998; McAdam et al. 2002; Noble et al. 2014). Phenotypes vary in a population as a product of the individual's genotype, the environment it experiences, and the combination of both (Kruuk 2004; Bolund et al. 2011). That is, trait variation is shaped by past selection, and how the environment currently impacts upon trait development (Hubbard et al. 2015). Morphological or life history traits are likely to be affected by a large number of genes (Falconer and Mackay 1996; Lynch and Walsh 1998) and harbour high levels of additive genetic variation and corresponding environmental variation (Houle 1992). The genetic basis of these phenotypic characteristics can be quantified indirectly by statistical inferences based on the degree of phenotypic similarities between relatives (Kruuk et al. 2000). However, similarities of traits between relatives can also be due to shared environmental conditions, as much as by heritable genetic effects (Kruuk and Hadfield 2007), making estimates of heritability upwardly biased if environmental effects are not taken into account. The phenotype of an individual does not solely depend on its genotype, but also on its interaction with the environment and maternal effects. This results in genotype-by-environment interactions (G×E) and maternal-by-environment interactions (M×E), which raise the possibility of trade-offs across environments. However, little is known about the relative importance of maternal variation and any interaction with the environment that then affect phenotypic trait expression.

How does it all come together?

In Chapter 1 I present evidence for behavioural traits that could lead to inbreeding avoidance. I measure mating preferences for novel mates (i.e. Coolidge effect) and explore whether the magnitude of the preference changes depending on whether the individual is familiar or a truly novel new mate. In Chapter 2 I explore the effects of mating with relatives. I highlight the importance of differentiating between a reduction in offspring fitness due to inbreeding depression and any reduction due to a decline in post-maternal investment.

In my third chapter I test if life history traits in an invasive fish show inbreeding

depression, and whether experiencing a stressful environment early in life could exacerbate any potential negative effects of inbreeding. I also explore if individuals are able to compensate for a poor start in life. Chapter 4 then explores whether a poor environment early in life affects adult male traits, namely sperm traits and genital size. In Chapter 5 I investigate how differences in inbreeding and the early dietary environment influence a key component of male fitness, namely reproductive success.

Chapter 6 focuses on parental effects and whether the inbreeding status of parents, their own early development, and their age shape offspring traits. I explore how these different factors interact, whether mothers and fathers affect their offspring in the same way, and whether parents affect their sons and daughters differently. Finally, in Chapter 7 I look at the heritability of a set of life history, morphological, and sexual traits, and I test for alternative ways in which environmental variation might shape trait variation.

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Chapter 1

The effects of familiarity and mating experience on mate choice in mosquitofish, *Gambusia holbrooki*

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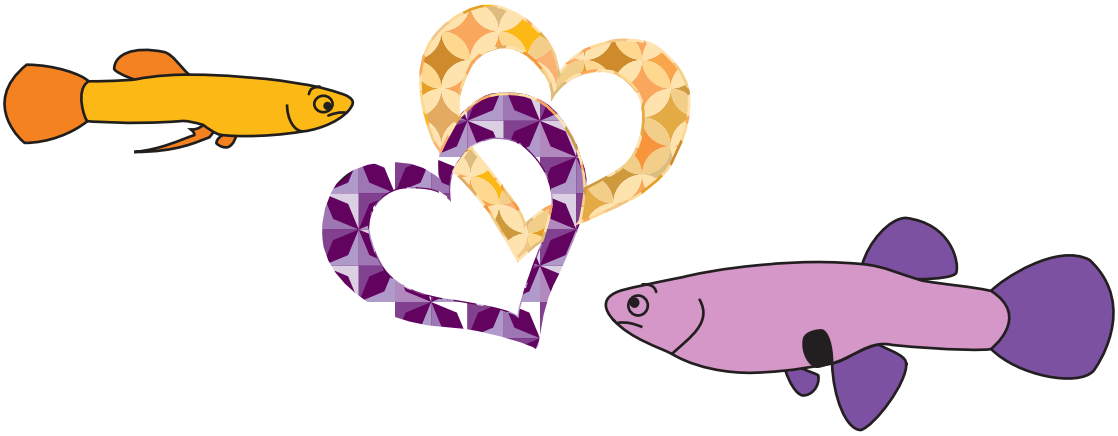

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Original Article

The effects of familiarity and mating experience on mate choice in mosquitofish, *Gambusia holbrooki*

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A preference to mate with novel partners has been shown for both males and females in a range of taxa. Preferences for novel mates may result from direct recognition of previous sexual partners, or from other cues that predict this, such as familiarity. Costs and benefits of mating with multiple mates differ for males and females. Despite this, few studies have tested whether the sexes differ in their preferences for novel mates. Here, we investigated whether males and/or females showed preferences for novel mates and whether this differed depending on the type of experience with a familiar mate (i.e., previously allowed to mate or allowed visual and olfactory exposure only) in the eastern mosquitofish (*Gambusia holbrooki*). We show that mosquitofish prefer to associate with novel fish and that there was no significant difference between the sexes in the strength of this preference if the choosing fish had previously had an opportunity to mate. In contrast, males and females that had not recently mated and were familiar due solely to visual and olfactory contact did not have a preference for novel mates. Our results suggest that there are likely to be benefits of mating with multiple partners for both males and females.

Key words: **Coolidge effect, mate preference, novel mate, remating, sexual selection.**

INTRODUCTION

Mating with multiple partners can be beneficial for both males and females. For females, polyandry can elevate the reproductive value of their offspring because postcopulatory mechanisms bias paternity toward more genetically compatible males (e.g., Tregenza and Wedell 2002) or toward better quality males (meta-analysis: Slatyer et al. 2012). Mating multiply can also increase the likelihood that females receive sufficient sperm to fertilize all their eggs (Pizzari 2002) or that females gain greater access to material resources provided by males, including nuptial gifts and parental care (review: Jennions and Petrie 2000). Although these direct benefits might be achieved by mating repeatedly with a single partner, potential variation in male quality means that these benefits are likely to be greater if females seek new, superior partners. For males, mating with more females allows greater fertilization opportunities and thus increases the total number of offspring sired. This is exemplified by the fact that males almost always have a positive Bateman gradient (i.e., the relationship between offspring number and the number of mates; Bateman 1948). Because the Bateman gradient is generally steeper for males than for females, it is often assumed that males gain more than females from mating with multiple partners (review: Kokko et al. 2012).

Individuals that prefer novel mates over previous mating partners can increase the likelihood that they mate with multiple partners, rather than repeatedly with the same individual (e.g., Archer and Elgar 1999). Increasing the mating effort invested into obtaining novel mates is a widespread phenomenon often referred to as the "Coolidge effect" (Dewsbury 1981). That is, sexual interest in a previous mating partner declines with each successive mating but is renewed when a novel individual is available (e.g., Koene and Ter Maat 2007; Steiger et al. 2008; Tlachi-Lopez et al. 2012). A decline in sexual interest, and subsequent recovery when exposed to a novel mate, has been measured as changes in latency to mate (e.g., Gershman and Sakaluk 2009), courting effort (e.g., Jordan and Brooks 2010), clutch size (e.g., LaDage et al. 2008), and in ejaculate quality and quantity (Dewsbury 1982; Wedell et al. 2002; Spence et al. 2013). For females, it could even involve postcopulatory processes such as biasing paternity toward a novel mate (e.g., Gershman 2009).

The most common test for a Coolidge effect is to offer an individual a choice between a prior mate and a novel potential mate with otherwise similar mating history (e.g., both are nonvirgins who have recently mated). Preferences for novel over previous mates have been reported

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for both females and males (Archer and Elgar 1999; Kelley et al. 1999; Eakley and Houde 2004; Ivy and Sakaluk 2005; Gershman 2009). To prefer novel mates minimally requires that the choosing sex recognizes and discriminates against familiar individuals with whom they might have mated (Griffiths and Ward 2006; Valero et al. 2009). The likelihood of mating multiply is further increased when individuals can actually recognize novel mates, possibly based on rare phenotypes (Zajitschek et al. 2006; Zajitschek and Brooks 2008) and/or based on the absence of self-referent cues that allow individuals to identify previous mates (Ivy et al. 2005; Steiger et al. 2008).

Strictly speaking, the Coolidge effect refers to a lower sexual response to a previous sexual partner, but sexual interest can also decline simply due to familiarity with an individual (e.g., Zajitschek et al. 2006; Jordan and Brooks 2010). This suggests that in some species, familiarity is a "rule of thumb" to indicate that an individual might have been a previous mate (see Tan et al. 2013). The ability to identify unfamiliar individuals can still offer a reproductive advantage, on average, even if some familiar individuals have not mated (e.g., Kelley et al. 1999).

Experimental studies of mate choice are essential to quantify the relative preference of each sex for novel mates. To date, most studies of the Coolidge effect have examined only a single sex per species (for exceptions, see Zajitschek et al. 2006; Ferkin et al. 2010; Mariette et al. 2010; Tan et al. 2013). Furthermore, taxonomic bias with regard to which sex research has focused on makes evaluation of sex differences in preferences for novel mates difficult. For instance, studies of male choice are more often carried out on small mammals (Dewsbury 1981; Pierce et al. 1992), whereas studies of female choice are more often conducted using insects, fish, and birds (Archer and Elgar 1999; Beguin et al. 2006; Zajitschek and Brooks 2008). Here, we experimentally tested for mating preferences of both sexes for novel versus familiar individuals in the mosquitofish (*Gambusia holbrooki*). We also tested whether the relative size of the potential novel mate had an effect on mate choice. In many fish, females prefer to mate with larger males (Head et al. 2013) and males also prefer to mate with larger females as it is positively correlated with fecundity (Andersson 1994; Casalini et al. 2013). In *G. holbrooki*, there is evidence for both male and female preferences for larger mates (e.g., males: Bisazza et al. 1989; Wong and McCarthy 2009; Mautz and Jennions 2011; Booksmythe et al. 2013 and females: Bisazza et al. 2001; Kahn et al. 2010, 2012).

To measure mating preferences, we initially performed 2-choice trials based on relative association time with a novel or previously encountered fish. We then conducted mating trials where these fish could interact freely. We had 5 aims:

(1) To test for a mating preference for novel mates (i.e., Coolidge effect).
(2) To determine if the magnitude of preferences for novel mates depended on whether the previously encountered fish was familiar (i.e., allowed only visual and olfactory exposure; not mated for more than 3 months prior mate exposure) or was a previous mate (i.e., allowed to interact freely; mated within 24 h to mate exposure). We expected the effect to be stronger if the choosing individual had already mated with the familiar fish.
(3) To test if the effect of familiarity versus actual mating differs between the sexes.
(4) To test for an effect of the relative size of the novel mate. We predicted a weaker preference for a novel mate if it was smaller than the familiar fish.
(5) To test whether association time in 2-choice trials predicts how males direct mating effort (copulation attempts) when individuals freely interact.

METHODS

The mosquitofish used in our study were the offspring of wild-caught fish collected in Sydney (33°48′50.14″S, 150°45′38.75″E) in November 2012 and February 2013. Fish were reared in the laboratory on a 14:10 h photoperiod at 28 °C and fed ad libitum with *Artemia nauplii* and commercial flakes. All the fish used were adults kept in large, same-sex holding tanks once they were old enough to be sexed. Females were male deprived for at least 3 months prior to mate choice tests to ensure that they had identical recent sociosexual histories.

All females presented to males as potential mates were marked with a small colored dot for visual identification using fluorescent elastomer (Northwest Marine Technology, Shaw Island, WA) injected subcutaneously behind the caudal fin. They had at least 4 days recovery before choice trials. We measured the standard length (SL = snout tip to base of caudal fin) of all fish. Fish from same-sex holding tanks were randomly assigned either as one of the stimulus pair or as a focal test fish. We did not match fish for size (males: 16.8–26.6 mm and females: 23.1–33.2 mm). Each fish was only used once as a potential mate or as a focal test fish whose mating preference was measured. All trios were unique.

Experimental design

To determine whether male and/or female mosquitofish showed preferences for novel partners, we allowed focal test fish to choose between 2 potential mates: one that they had prior experience with and one that they had no experience with. We applied 2 treatments to fish with whom they had prior experience: mated or familiar. For the mated treatment, the focal fish chose between a stimulus fish that they had previously been allowed to interact freely with (i.e., kept for 24 h together in a 6-L tank) and a stimulus fish that had experienced the same protocol with another fish. For simplicity, we refer to these test fish as "mated." This is highly probable given the high rate at which males attempt to inseminate females (see Wilson 2005), but we did not directly confirm that mating occurred. For the familiarity treatment, the focal fish chose between a stimulus fish with whom it had previously had visual and olfactory contact (i.e., kept for 24 h together in a 6-L tank separated by a mesh partition) and a stimulus fish that had experienced the same protocol but with another fish. In addition to differing in whether they were mated or familiar, the treatments also differed in mating history. That is, fish from the mated treatment had likely mated in the previous 24 h, whereas fish from the familiar had not mated for at least 3 months prior to trials.

Association time choice trials

We performed 40 trials per treatment (N = 160; 2 sexes and 2 treatments) with observations lasting 10 min (see McLaughlin and Bruce 2001; Simcox et al. 2005; Mariette et al. 2010). After 24 h being kept in either the mated or familiar treatment, fish were individually transferred to separate 1-L tanks and left for 30 min. They were then transferred to the test tank. The test tank was a 16.6-L (38 × 19 × 19 cm) glass aquarium divided into 3 sections: 2 end sections (5 × 19 × 19 cm) held the stimulus fish and a central section (28 × 19 × 19 cm) held the test fish. The sections were

each separated by a removable opaque screen and a mesh screen. A novel fish was randomly assigned to an end compartment at the start of each trial. After a 5-min acclimation period, we removed the opaque screens, so that the fish were in visual and olfactory contact and began the trial. We calculated the test fish's mating preference based on association time (i.e., time spent within 4 cm of the end compartment facing a potential mate). The relative time that the test fish spent at the end compartment housing the novel fish was calculated. For a trial to be included in our analysis, the test fish had to visit both choice zones (18 of 178 trials were discarded for this reason).

Free-swimming mating trials

To validate the use of association time to measure male mating preferences, we conducted mating trials where test fish were allowed to interact freely with the stimulus fish. Immediately after the choice trial ended, we lifted both mesh screens to allow the 3 fish to interact. We then recorded 1) the male association time with each female (defined as occurring when he was oriented toward a female and within one body length) and 2) the number of mating attempts per female, defined as gonopodium thrusts made after being initially positioned below and slightly behind her. We only conducted mating trials for test males because mosquitofish mating behavior, which consists of males harassing females, makes it difficult to measure female choice when fish freely interact: both males continuously swim alongside the female unless one drives the other away. Males do not court females but instead position themselves below the females in an attempt to transfer sperm through their gonopodium, an organ modified from the anal fin (Bisazza and Marin 1991). We performed 40 trials per treatment ($N = 80$) with each observation lasting 10 min. Data were collected using a handheld event recorder.

To verify the accuracy of data collected directly during the trials, we recorded them with a high-speed video camera. We then compared the data obtained directly with that from analysis of the recordings (collected by an observer blind to the treatment or identity of the novel fish). There was a strong correlation between data collection methods for 1) difference in association time with females ($r = 0.540$, $P = 0.002$, $N = 30$) and 2) number of mating attempts ($r = 0.792$, $P < 0.001$, $N = 60$). Given that these methods of data collection gave similar results, we decided to use data collected directly during the trials as we felt the presence of 3D information made this method more accurate.

Statistical analysis

We analyzed the relative time the test fish spent with each mate in the choice trials as the proportion of association time spent with the novel fish in a generalized linear model (GLM) with quasi-binomial error using the cbind function in R 3.0.2 software (R Development Core Team 2012; i.e., including information on the total amount of time each fish spent in association with potential mates). We included sex, treatment, and the interaction between these factors as fixed factors in the model. We also included the size difference between the potential mates (novel minus familiar or mated) as a covariate, as size is known to be important in mate choice in mosquitofish. We did not include any other 2- or 3-way interaction terms in the model because we decided a priori that these were not the aim of this study (their inclusion does not, however, change the key findings we report). We included which side the novel fish was on to control for any inadvertent side bias. To directly test for a preference for novel mates in each treatment by each sex, we then followed this analysis with 4 separate GLM models (as above) that only included size difference as a predictor. Here, we were interested in the estimate of the intercept (i.e., when stimulus fish are the same size, do focal fish show a preference for a novel partner?). Given the clear prediction, we used 1-tailed tests (i.e., we predicted that the intercept was greater than 0). An intercept of 0 corresponds to 50% of the time spent with each mate ($\ln(p/[1-p])$, where p = proportion of time with the novel mate). If the trend was in the opposite direction, we report the 2-tailed P value. Unless otherwise noted, 2-tailed P values are reported.

To examine male preferences in the free-swimming mating trials, we used the proportion of copulation attempts directed at the novel female as the dependant variable in a GLM with quasi-binomial error using the cbind function. Treatment, size difference, and their interaction were included as fixed factors in the model. We excluded 12 of 80 trials because the focal male did not attempt to mate with either female so they were uninformative. We again followed this analysis with 2 complimentary GLM models with size difference as the sole predictor specifically to test for a preference for novel partners within each treatment by testing whether the intercept was significantly greater than 0 (description above).

Finally, we tested whether we could predict the relative number of male attempts directed at the novel female in the free-swimming mating trials using the relative time spent associating with her during the choice trials. We used the proportion of the male attempts directed at the novel female as the dependant variable in a GLM with quasi-binomial error using the cbind function. The treatment, the proportion of association time spent with the novel female during the choice trial, and their interaction were included in the model as fixed factors.

RESULTS

Association time choice trials

There was no significant difference between males and females in the proportion of time spent in association with the novel fish. There was also no interaction between the sex of the test fish and whether or not the previously encountered fish was a familiar or previously mated fish (i.e., familiar/mated treatment). However, test fish spent significantly more time associating with a novel fish when the previously encountered fish was a former mate, rather than only a familiar fish (Table 1). Both males and females spent significantly more than 50% of their time in association with a novel male in the mated treatment trials ($GLM_{\text{males mated}} = 0.639 = 65.5\%$, $t_{(39)} = 2.676$, $P_{\text{1-tail}} = 0.006$; $GLM_{\text{females mated}} = 0.481 = 61.8\%$, $t_{(39)} = 2.253$, $P_{\text{1-tail}} = 0.015$). The 2 intercepts (i.e., estimates of the proportion of time spent with a novel mate) did not differ significantly from each other: $t_{78} = 0.699$, $P = 0.487$, Figure 1. There was, however, no preference for novel mates in the familiar treatment ($GLM_{\text{males familiar}} = -0.100 = 47.5\%$, $t_{(39)} = -0.363$, $P = 0.719$; $GLM_{\text{females familiar}} = -0.153 = 46.2\%$, $t_{(39)} = -0.673$, $P = 0.505$; Figure 1).

Free-swimming mating trials

We found a highly positive correlation between the relative time that a male spent close to the novel female during the mating trial and the proportion of his mating attempts directed toward the novel female ($r = 0.811$, $N = 68$, $P = 0.01$). Mating attempts are a direct measure of male sexual interest, so we have only analyzed

Table 1

Results of GLMs (quasi-binomial error) for the response variables: proportion of time with novel fish in choice trials and proportion of male mating attempts directed at the novel fish during mating trials

Response variable and factors	df	*F*	*P*
Proportion of time with novel fish			
Sex	1,158	0.052	0.820
Treatment	1,157	9.942	**0.001**
Size difference	1,156	2.160	0.144
Side	1,155	4.455	**0.036**
Sex × treatment	1,154	0.669	0.415
Proportion of male attempts directed at novel fish			
Treatment	1,66	0.105	0.748
Size difference	1,65	14.427	**<0.001**
Treatment × size difference	1,64	11.934	**0.001**

The main effects are sex (male/female), treatment (mated/familiar), size (novel—familiar or mated), and side (novel fish on the left or right). df, degrees of freedom. Bold values represent significant values.

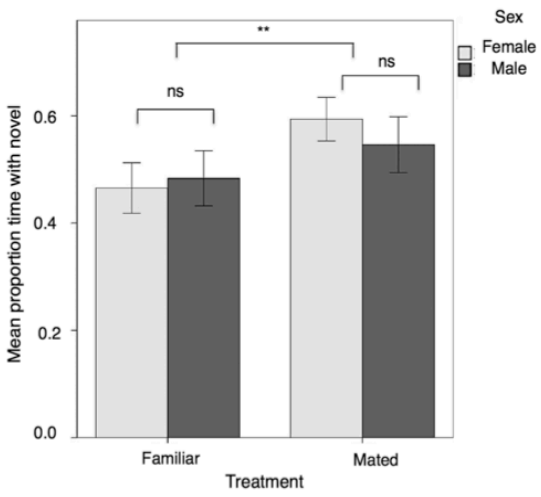

Figure 1

Mean ± SE proportion of association time spent with the novel individual given the previously encountered fish was assigned to the mating or familiar treatment. Significant differences ($P < 0.05$) are represented by **. Note that these are raw means that do not account for size difference between the novel and familiar fish and that treat each data point equally (i.e., unlike the GLM in Table 1, there is no weighting by the total time the focal fish spent in association with potential mates).

this measure of male choice. Unexpectedly, the effect of relative female size on the proportion of attempts directed at the novel female depended on whether or not the male had previously had the opportunity to mate with her (Table 1). Males that were only familiar with the previously encountered female, but had not had a chance to mate with her, were unaffected by the female size difference ($F_{1,32} = 0.001$, $P = 0.982$). In contrast, if there had previously been an opportunity to mate, then the male was more likely to direct his mating attempts toward the larger of the 2 females ($F_{1,34} = 6.509$, $P = 0.016$) (Figure 2). We did not find a significant preference for novel mates in either treatment although there was a marginally nonsignificant trend in the mated treatment ($GLM_{mated} = 0.246 = 56.1\%$, $t_{(34)} = 1.606$, $P_{1\text{-tail}} = 0.059$; $GLM_{familiar} = 0.010 = 52.5\%$, $t_{(32)} = 0.060$, $P_{1\text{-tail}} = 0.476$).

Predicting male courtship by association time

The proportion of time a male spent in association with the novel female during the choice trials predicted the proportion of his mating attempts subsequently directed at her during the free-swimming mating trials ($F_{1,66} = 16.386$, $P < 0.001$). There was no difference between the familiar and mated treatments in the strength of this relationship ($F_{1,64} = 0.026$, $P = 0.873$; Figure 3). Association time in 2-choice trials was, therefore, a good general predictor of actual male mate choice as measured by mating attempts.

DISCUSSION

We found that, under certain conditions, mosquitofish (*G. holbrooki*) exhibited a significant preference for novel individuals (i.e., Coolidge effect). Contrary to expectations based on a stronger relationship between the number of mates and reproductive success in males than females (i.e., males have a steeper Bateman gradient; Kokko et al. 2012), there was no significant differences between the sexes in the strength of the observed Coolidge effect (Table 1). Others have, however, argued that higher costs per mating for females than males and ongoing benefits of repeated mating with the same female when there is sperm competition could actually generate stronger selection on females than males to discriminate against previous mates (see Mariette et al. 2010). Thus, the relationship between the number of mates and offspring number alone might not account for the Coolidge effect. It is worth noting that Bateman gradients are often short-term estimates of the fitness returns of mating and, therefore, ignore any longer-term costs associated with an increased number of mates (e.g., future effects on mortality, fecundity, or sexual attractiveness; Kokko et al. 2012).

In mosquitofish, whether males or females exhibited a preference for novel mates depended on whether the alternate mate was an individual that the test fish had previously encountered (i.e., familiar treatment) or one with whom they had actually had the opportunity to mate (i.e., mated treatment). When the alternate fish was from the familiar treatment, neither sex showed a significant preference for a novel partner. This result is consistent with our prediction that the Coolidge effect should be stronger when mating had occurred. Familiarity alone should not affect the value of a potential mate.

Alternatively, the difference in the preference for novel mates seen between our mated and familiar treatments may be due to differences in the recent mating history of test (and stimulus) fish. All fish in the mating treatment had recently mated, but in the familiar treatment, they had not. In some species, virgin females are less choosy than mated females (e.g., Pitcher et al. 2003), and likewise, males with smaller sperm reserves following a recent mating might be more choosy than those with full reserves (Bukowski et al. 2001; Bateman and Ferguson 2004). The lack of preference for novel partners in our familiar treatment might therefore have arisen because focal fish had not mated in the previous 3 months and were therefore generally less choosy than those in the mated treatment. To our knowledge, only a few studies have teased apart the effects of familiarity and recent mating history on mate choice for novel mates (e.g., Zajitschek et al. 2006; Tan et al. 2013). Such tests are necessary to confirm with certainty that there is a direct effect of experience type (i.e., familiar only vs. mate) on preferences for novel individuals. It should be noted, however, that the mating history of potential mates was identical within each treatment, so there is still clear evidence for a Coolidge effect in the mating treatment.

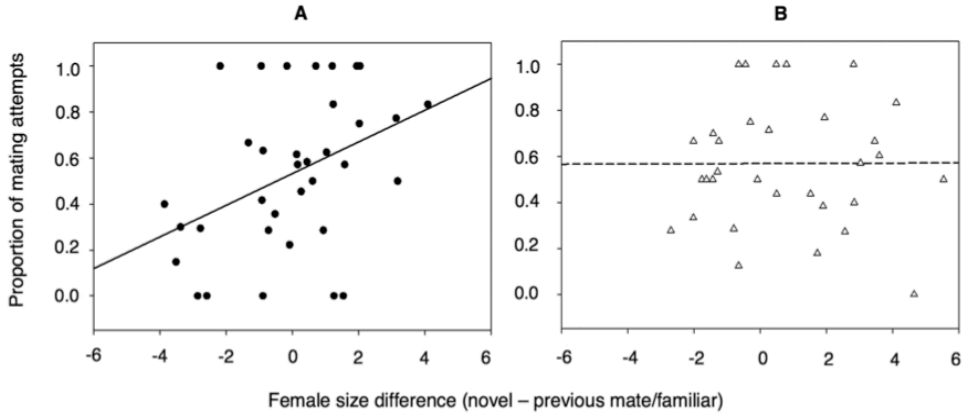

Figure 2
The association between the female size difference (novel − previous mate/familiar mate) and the proportion of male mating attempts directed at the novel female during mating trials. (A) Mated treatment. (B) Familiar treatment. Note that the regression line drawn is based on treating each data point equally (i.e., unlike the GLM in Table 1, there is no weighting by the total number of times that focal males attempted to mate).

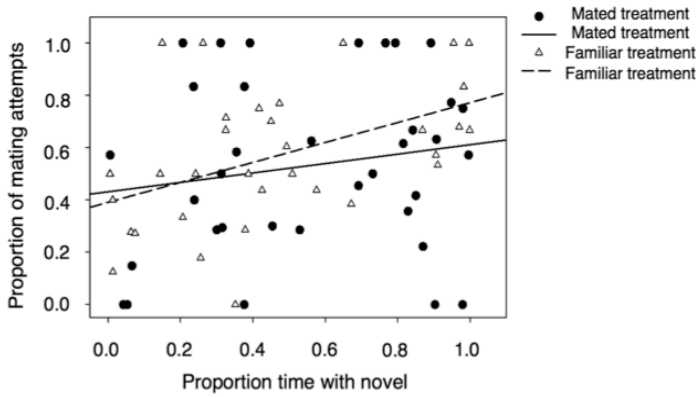

Figure 3
The relationship between the proportion of time spent with the novel female during choice trials and the proportion of male mating attempts directed at her during mating trials. Note that the regression lines shown are based on treating each data point equally (i.e., unlike the GLM in the text, there is no weighting by the total number of times that focal males attempted to mate).

A comparison with guppies

Poeciliid fish are the subject of intense investigation by those studying sexual selection (Evans et al. 2011), but relatively few studies have investigated the Coolidge effect. Most of these studies are on guppies (*Poecilia reticulata*), so they are our main source of comparison to *G. holbrooki*. Although the mating systems of these 2 species differ (male guppies court and coerce females, whereas male mosquitofish only coerce females), several studies have shown that female mosquitofish are able to exert mate choice by preferentially associating with certain males and thus increasing the likelihood of insemination by these males (e.g., Bisazza et al. 2001; Pilastro et al. 2003; Kahn et al. 2010, 2012).

In guppies, females generally prefer novel over familiar males (e.g., Zajitschek et al. 2006; Mariette et al. 2010). This is consistent with a trend for female guppies to prefer males with phenotypes that they are unlikely to have encountered previously ("rare male effect," see Zajitschek and Brooks 2008; Hughes et al. 2013). This mating preference has been attributed to inbreeding avoidance because unfamiliar males with unusual color patterns are more likely to be unrelated (Kelley et al. 1999; Mariette et al. 2006; Zajitschek et al. 2009). Our result that a preference for novel partners only exists when focal fish have mated with the familiar fish suggests that inbreeding avoidance is unlikely to be the driver of preferences for novel partners in mosquitofish. Furthermore, the "rare male effect" may be less likely to contribute to the patterns we see here than is the case in guppies because guppies show extreme color pattern polymorphism, whereas mosquitofish do not.

Although we have documented male and female preferences for novel mates in mosquitofish, the adaptive significance of these preferences is unclear. For males, the benefits of mating with multiple partners are straightforward—more mates means more offspring. For females, reasons for preferring novel mates are less clear. First, females may gain indirect benefits of mating with multiple partners if it allows females to bias paternity toward males that sire higher quality offspring (e.g., Evans and Magurran 2000). However, general evidence for this benefit is weak (meta-analysis: Slatyer et al.

2012). Second, females may gain a fertilization benefit. This is plausible if male sterility is common or if genetic incompatibility between males and females leads to a failure to fertilize eggs (Pizzari 2002; Wedell et al. 2002). Assessing whether the patterns we see here are driven by such fertility benefits is difficult as there is little information available about variation in male fertility or genetic incompatibility in mosquitofish. As such, this remains a potentially important form of selection that deserves further investigation. Third, females may avoid males that have previously harassed them (Bisazza et al. 2001; McLaughlin and Bruce 2001). This could explain why females only showed a preference for novel males in our mated treatment where fish were allowed to fully interact as opposed to the familiar treatment where males could not engage in coercive behavior. Finally, direct interactions between fish (i.e., during the mating treatment) may provide additional cues that increase the ease with which a female can recognize familiar males (Hughes et al. 1999). If true, females in the familiar treatment may have been less capable of discriminating between novel and familiar males than those in the mated treatment. In the choice trials, both males and females showed a significant preference for associating with a novel mate, but only if they had an opportunity to mate with the previously encountered fish. In the free-swimming mating trials, however, there was only a nonsignificant tendency for males to attempt to mate with the novel female ($P = 0.059$ from the GLM).

In guppies, there is contradictory evidence as to whether males prefer novel, unfamiliar females. For example, no such preference was reported by Zajitschek et al. (2006), but one was reported by Kelley et al. (1999), Mariette et al (2010), and Jordan and Brooks (2010). Mariette et al. (2010) showed, however, that females showed a stronger preference for unfamiliar mates than males did and suggested that this difference could result from indirect effects of female behavior (e.g., preferences or receptivity) on male behavior. Indirect effects of female behavior on male behavior may also explain differences in male preferences for novel mates that we see here between trial types, as male preference for novel females was lower when males and females were allowed to interact freely.

In our study, one unexpected finding from the free-swimming mating trials was that males that had previously had an opportunity to mate with the familiar female directed a significantly higher proportion of their mating attempts to whichever of the 2 females was the larger. In contrast, there was no effect of relative female size on the proportion of mating attempts directed at each female by males that had only had visual and chemical contact with the familiar female (Figure 2). Males then tried to mate with larger females even when they had previously mated with them. It is possible that males did not prefer mating with novel females, but only bigger females, or even that mating with any female takes precedence over any other discrimination (Sievers and Magurran 2011).

Validating association choice tests

Few studies of mate choice based on association times in setups equivalent to our choice trials have validated that association time during choice trials predicts mating behavior when fish can freely interact (review: Jeswiet and Godin 2011). We found that the proportion of time males spent with a novel female during choice trials was significantly positively related to the proportion of mating attempts directed at the same female during mating trials when fish could freely interact. Our study is, therefore, in agreement with studies of other Poeciliid fish in showing that association time is a reasonable proxy for actual mating behavior (guppies: Jeswiet and Godin 2011; swordtails: Walling et al. 2010). This strengthens the interpretation of previous studies of male choice in mosquitofish based on association time (e.g., Bisazza et al. 1989; Mautz and Jennions 2011; Callander et al. 2012; Booksmythe et al. 2013).

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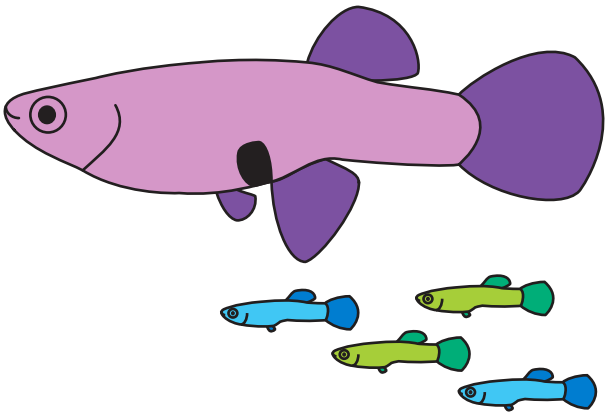
Chapter 2

Evidence for inbreeding depression in a species with limited opportunity for maternal effects

Ecology and Evolution 5(7): 1398-1404



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Evidence for inbreeding depression in a species with limited opportunity for maternal effects

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Keywords
Lecithotrophic, maternal investment, offspring fitness, relatives.

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Abstract

It is often assumed that mating with close relatives reduces offspring fitness. In such cases, reduced offspring fitness may arise from inbreeding depression (i.e., genetic effects of elevated homozygosity) or from post-mating maternal investment. This can be due to a reduction in female investment after mating with genetically incompatible males ("differential allocation") or compensation for incompatibility ("reproductive compensation"). Here, we looked at the effects of mating with relatives on offspring fitness in mosquitofish, *Gambusia holbrooki*. In this species, females are assumed to be nonplacental and to allocate resources to eggs before fertilization, limiting differential allocation. We looked at the effects of mating with a brother or with an unrelated male on brood size, offspring size, gestation period, and early offspring growth. Mating with a relative reduced the number of offspring at birth, but there was no difference in the likelihood of breeding, gestation time, nor in the size or growth of these offspring. We suggest that due to limited potential for maternal effects to influence these traits that any reduction in offspring fitness, or lack thereof, can be explained by inbreeding depression rather than by maternal effects. We highlight the importance of considering the potential role of maternal effects when studying inbreeding depression and encourage further studies in other Poeciliid species with different degrees of placentation to test whether maternal effects mask or amplify any genetic effects of mating with relatives.

Introduction

Mating with close relatives often reduces offspring fitness (Keller and Waller 2002). This can take the form of a reduction in offspring birth weight, survival, or reproductive success, as well as resistance to disease, predation, and environmental stress (Keller and Waller 2002; Frommen et al. 2008). The decrease in offspring fitness resulting from mating with close relatives is often attributed to inbreeding depression (Charlesworth and Charlesworth 1987; Falconer and Mackay 1996). Inbreeding depression results from an increase in the levels of homozygosis (Keller and Waller 2002; Frommen et al. 2008) and has been explained by two main hypotheses. The overdominance hypothesis, where heterozygotes, which are assumed to be superior to homozygotes, decrease in frequency, and the partial dominance hypothesis where the unmasking of deleterious recessive alleles due to greater homozygosity reduces fitness (Charlesworth and Charlesworth 1987). However, inbreeding depression is not the only explanation for differences in offspring fitness when mating with close relatives rather than unrelated individuals.

Maternal investment in offspring in response to male traits is known to have important effects on offspring phenotypes (Kindsvater and Alonzo 2014). This means that variation in offspring traits, particularly those expressed early in life, may result from variation in maternal investment (i.e., maternal effects) rather than being solely attributable to offspring genotype. Mothers can differentially allocate resources into offspring to maximize their fitness (Sheldon 2000). This is widely associated with greater maternal investment into offspring sired by more attractive males, who possess generally preferred traits (e.g., large ornaments; Arct et al. 2010; Horvathova et al. 2012). It follows that differential allocation by females may also be influenced by the relatedness of their mating partner (Lihoreau et al. 2008) as genetically similar males are generally considered to be less attractive

mates because of the potential costs of inbreeding (Tregenza and Wedell 2000). Females may therefore be expected to reduce investment in offspring that are sired by closely related males (e.g., Sardell and DuVal 2014). Alternatively, females could partially compensate for the lower quality of their offspring by providing more resources when mating to nonpreferred or genetically incompatible mates (Ratikainen and Kokko 2010). If present, maternal effects may enhance (for differential allocation) or mask (for reproductive compensation) the potentially negative genetic effects of mating with a relative.

Early life-history traits such as embryo survival, number, quality, and the viability of offspring (Bernasconi et al. 2004; Frommen et al. 2008) are closely related to fitness (DeRose and Roff 1999; Janicke et al. 2014) and, as such, often suffer from inbreeding depression (Roff 1998; DeRose and Roff 1999). However, these are the same traits that are most likely to be influenced by maternal effects (Wolf and Wade 2009; Kindsvater and Alonzo 2014). Consequently, it is important for studies investigating how mating with relatives influences offspring performance to consider, and ideally control for, maternal effects to avoid potentially inaccurate measures of inbreeding depression.

Here, we examine the effects of mating with relatives on offspring fitness in mosquitofish (*Gambusia holbrooki*), a species with limited opportunity for post-mating maternal effects. They are small fish that live in streams and ponds (Pyke 2005) with seasonally fluctuating water levels, so they are often exposed to stochastic reductions in population size, especially during dry seasons (Scribner et al. 1992; Griffiths and Magurran 1997). This makes them vulnerable to the risk of inbreeding. Furthermore, mosquitofish are lecithotrophic (i.e., allocate resources for embryo development to eggs before fertilization), which limits the opportunity for females to differentially allocate resources toward offspring after mating (i.e., matrotrophy; Ojanguren et al. 2005; Pollux et al. 2014). There is limited evidence of transfer of nutrients such as amino acids and metals in other species of mosquitofish (Marsh-Matthews et al. 2005, 2010; Cazan and Klerks 2014) that suggests post-fertilization transfer from mother to embryos. Although this means that there is the potential for maternal effects to confound those directly due to inbreeding depression, the lack of evidence for an increase in offspring mass between the egg and birth stage strongly suggests that transfer of nutrients does not generally occur in *Gambusia holbrooki* (Pollux et al. 2014).

We looked at the effects of mating with a sibling on several reproductive and early life-history traits. We examined offspring number, offspring size, gestation period, and early offspring growth. If we assume, based on the lack of evidence for matrotrophy, that eggs are fully provisioned prior to mating, we predicted that genetic effects of mating with relatives would most likely influence the number of offspring (via effects during fertilization or embryo development), as well as their size at birth and their growth after birth. On the other hand, we predicted that maternal effects are likely to influence the proportion of females breeding and gestation time (i.e., females can determine if and when to fertilize eggs).

Materials and Methods

Origin and maintenance of fish

Our laboratory stock of mosquitofish originated from 151 wild-caught females collected in Canberra, Australia in February and March 2013. F_1 generation offspring were kept in single sex tanks under a 14:10 h photoperiod at 28°C and fed ad libitum with *Artemia nauplii* and commercial flakes.

Experimental design

To create our parental generation, we set up 150 unique male–female pairs that were randomly created from the F_1 laboratory stock (described above). From these, we obtained 58 outbred F_2 full-sib families that were used to examine the effects of mating with relatives on female reproductive effort and early life offspring performance. We used a fully balanced block design that involved mating individuals from two families (e.g., A and B). Brothers and sisters from full-sibling families were paired to create inbred offspring (AA and BB) and outbred offspring by the reciprocal crossings of males and females from each family (AB and BA; Fig. 1). We set up multiple

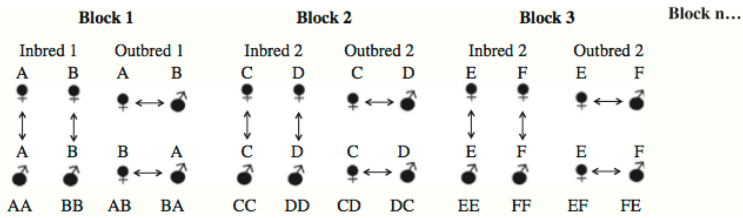

Figure 1. Block design for mating F_2 families to create inbred vs outbred fish. Each block involved between one and four full sisters and one male per cross-type from two families (A and B in block 1, C and D in block 2, E and F in block 3, and so on). Arrows indicate matings.

females (one to four full sisters) per cross-type (AA, AB, BA, BB). Within each block, the same potential number of females contributed to each cross-type. Only one male contributed to each cross-type so that within each block the offspring of each cross-type were either full siblings or paternal half siblings. Males and females were placed together for 1 week to allow mating. Females were then placed in individual 1-L tanks and allowed 6 weeks to give birth. They were checked for offspring twice daily. We set up 29 blocks yielding a maximum total of 58 inbred families and 58 outbred families. We recorded the age and size (standard length, SL in mm) of each female on the day she gave birth, the gestation time, the number of offspring, the size of offspring at birth, and their size 1 week later. To measure female size, females were anaesthetized by submersion in ice-cold water for a few seconds to reduce movement and then photographed alongside a microscopic ruler (0.1 mm gradation). To measure offspring size, fry were placed in a plastic dish (27 × 27 mm) with 2 mm depth of water to restrict movement and a scale at the bottom. All offspring were photographed within 18 h of birth.

Statistical analysis

We tested for a difference in reproductive success between females mating with a related or an unrelated male by comparing the proportion that gave birth within 6 weeks of the mating period using a chi-squared test. When testing for an effect of mating with relatives on gestation time and the number of offspring produced, we only included first broods by females that gave birth during the first 6 weeks. This avoids any confounding effect of a change in brood size with brood order (Larsen et al. 2011). These analyses were based on a single value per brood. To test for an inbreeding effect on size at birth and growth rates (size at 1 week of age − size at birth), we included the data from each individual offspring that the female gave birth to. Cross-types AA and BB were classified as inbreeding, while AB and BA were classified as outbreeding.

Female reproductive effort

We used generalized linear mixed-effect models (GLMM) with Gaussian error to test for fixed effects of treatment (related or unrelated male), female age, and female size on gestation time, number and size of offspring, and the growth rate of offspring with the *lmer* function using the *lme4* package in R 3.0.2 software (R Development Core Team 2012). We included the female's family identity as a random effect when testing for effects on gestation time and number of offspring. We included the female's individual identity as a random factor when testing offspring size and growth (as we measured multiple fry per female). We treated maternal age and size as independent predictors because they were uncorrelated ($r = -0.027$, $P = 0.716$, $N = 179$; age range: 82–141 days, size: 22.76–31.25 mm).

Inbreeding coefficient

We calculated the standardized coefficient of inbreeding δ (Lande and Schemske 1985) as the percentage change with inbreeding: (outbred trait value − inbred trait value) / outbred trait value. A negative value indicates that inbred individuals had a larger value for the trait, interpretation of which depends on the direction of selection on the trait.

Results

There was no difference in the proportion of females producing broods when mated with either a related or unrelated male. From 162 females that mated with their brother, 79.6% gave birth, while from 147 females mated with an unrelated male, 77.5% gave birth ($\chi^2 = 0.198$, df = 1, $P = 0.656$). From 309 females that could have produced broods, 199 were used for analyses of first broods produced within 6 weeks of mating (112 mated with a brother; 87 with an unrelated male).

Female reproductive effort

The number of offspring a female gave birth to (range: 1- 15) was affected by whether or not she mated with a related male (Fig. 2). Females mated to their brother gave birth to significantly fewer offspring than those mated to an unrelated male (an inbreeding coefficient of δ = 14.5%; Table 1). The number of offspring in the brood was significantly negatively related to the female's age, but significantly positively related to her size (Table 1).

In contrast, we found no evidence that mating with related males affected the gestation time of females, the size of offspring at birth (range: 6.61–9.21 mm), or early offspring growth. Nor did we find any effect of female size or age on any of these traits. Further, we found no repeatable difference in gestation time among families (Table 1).

Discussion

Variation in traits expressed in offspring can be attributable to both parental effects and offspring genotype. For example, life-history traits related to female reproductive effort are a maternal character but they can also affect

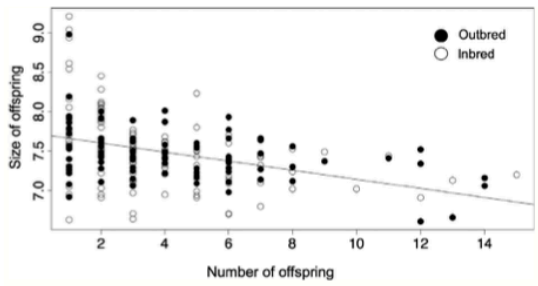

Figure 2. The association between number and size of outbred and inbred offspring.

offspring fitness (Bernardo 1996; Fischer et al. 2006). When assessing whether mating with relatives causes inbreeding depression, maternal effects from differential allocation or reproductive compensation could exacerbate or mask potential genetic effects. In the present study, we found that mating with a relative (full sibling) in *Gambusia holbrooki* significantly reduced the number of offspring at birth (δ = 14.5%). There was, however, no significant decrease in the likelihood of breeding, no increase in gestation time (δ = 4.2%), and no reduction in the size (δ = 0.2%) or growth (δ = 2.1%) of the resultant offspring. Given the reproductive physiology of *G. holbrooki* (fully yolked eggs are produced prior to mating), there is no obvious mechanism for post-mating maternal effects on offspring size or growth, and maternal effects on offspring number and gestation time seem unlikely. It has, however, been suggested that mosquitofish are incipient matrotrophic rather than lecithotrophic organisms based on transfer of metals from mothers to offspring (Cazan and Klerks 2014), so we cannot definitively exclude the possibility that there are subtle maternal effects. Nonetheless, the decline in offspring dry weight from the egg to birth stage in *G. holbrooki* suggests that there is no transfer of nutrients to offspring (Pollux et al. 2014).

The smaller brood size of females mated to a related rather than an unrelated male has several potential explanations. First, sperm allocation toward related and unrelated females might differ (Firman and Simmons 2008; Lewis and Wedell 2009). However, it is unlikely that this explains our findings because males did not choose between females, and previous studies on mosquitofish (Head et al., *in press*) and more generally (Barry and Kokko 2010) show that males are rarely choosy when encountering females sequentially. Further, even very low sperm transfer is still likely to provide sufficient sperm to fertilize a full clutch (Bisazza and Marin 1991; Johnson et al. 2010). Second, females might decide not to fertilize all their eggs when mating with males of low compatibility (e.g., Olsson et al. 1996; Birkhead 1998). This is unlikely for several reasons: (1) Our experimental design reduced the potential for choice – females were virgins and previous work on Poeciliids has shown that virgins are not choosy with respect to mate quality (Pitcher et al. 2003), (2) There is little evidence of mate choice for unrelated males in Poeciliids (e.g., Pitcher et al. 2008; Ala-Honkola et al. 2010), but see (Kelley et al. 1999; Zajitschek and Brooks 2008) for studies showing male mate preferences based on familiarity and (Hain and Neff 2007) showing kin recognition in Poeciliids), and (3) If females differentially used

Table 1. Results of GLMs (Gaussian error) for the response variables: gestation time, number of offspring, size of offspring, and growth of offspring of females mated to related and unrelated males. Inbreeding coefficient (% change with inbreeding). Bold values represent significant values.

							Mean ± SE (*N*)		
Response	Predictor	β	SE	df	t	P	Inbred	Outbred	δ
Gestation time (days)	Intercept	28.756	17.224	101.150	1.669	0.098	33.67 ± 0.794 (112)	32.33 ± 0.883 (87)	−4.145
	Treatment	1.228	1.204	159.180	1.020	0.309			
	Female size	0.230	0.532	132.770	0.432	0.667			
	Female age	−0.028	0.083	70.880	−0.343	0.733			
Number of offspring	Intercept	−4.941	6.125	112.740	−0.807	0.422	3.83 ± 0.256 (112)	4.48 ± 0.344 (87)	14.509
	Treatment	−1.003	0.404	154.160	−2.481	**0.014**			
	Female size	0.671	0.186	143.070	3.599	**<0.001**			
	Female age	−0.065	0.030	77.000	−2.180	**0.032**			
Size of offspring (mm)	Intercept	7.030	0.783	101.800	8.975	<0.001	7.352 ± 0.029 (212)	7.368 ± 0.016 (590)	0.217
	Treatment	0.021	0.069	135.200	0.310	0.757			
	Female size	0.003	0.024	121.700	0.138	0.890			
	Female age	0.003	0.004	85.940	0.763	0.448			
Growth of offspring (mm in first week)	Intercept	3.104	1.164	99.150	2.666	0.009	3.633 ± 0.045 (172)	3.560 ± 0.028 (501)	−2.050
	Treatment	0.106	0.094	140.540	1.128	0.261			
	Female size	0.024	0.036	114.280	0.680	0.498			
	Female age	−0.003	0.005	83.500	−0.589	0.557			

sperm, this should increase their gestation time, and/or affect the proportion of females breeding. This did not occur. Females cannot provision eggs after fertilization, and lack superfetation (Ojanguren et al. 2005; Pollux et al. 2014), so there is no immediate benefit of discriminating against a related male's sperm (e.g., Pitcher et al. 2008). In short, there is no obvious adaptive explanation why females would partially fertilize a clutch.

Third, the most plausible explanation for females having fewer offspring when mated with related males is reduced fertilization success (i.e., low sperm survival due to sperm–female tract or egg interactions) and/or inbreeding depression lowering embryo survival (Pitcher et al. 2008; Johnson et al. 2010). In general, the evidence for a negative effect of mating with a related male-on-female reproductive effort is inconclusive: some studies report fewer offspring or eggs (e.g., Pitcher et al. 2008; Johnson et al. 2010), but others do not (e.g., Simmons et al. 2006; Ala-Honkola et al. 2009). However, based on studies of other Poeciliids, inbreeding depression for embryo viability is most likely to explain why *G. holbrooki* had fewer offspring after a full-sib mating (Pitcher et al. 2008; Johnson et al. 2010).

Offspring size at birth is under directional selection as larger offspring tend to be more competitive and survive better in stressful environments (Smith and Fretwell 1974; Simmons and Garcia-Gonzalez 2007). Larger offspring also tend to become adults with above average reproductive success (e.g., Czesak and Fox 2003). We did not, however, find any evidence of inbreeding reducing offspring size at birth or post birth growth, even though this should occur if higher homozygosity reduces the physiological efficiency with which offspring convert resources (i.e., egg yolk then *Artemia*) into body mass. One explanation for a lack of inbreeding depression is that offspring with bad genetic combinations died before birth. This explanation is also consistent with fewer offspring being born to females who mated with a brother.

In our experiment, males and females were allowed 1 week to interact and mate. We predicted that if females avoid mating with related males that those paired with their brother would take longer to mate and/or refrain from fertilizing their eggs and therefore would take longer to give birth. This did not occur. There is conflicting evidence for effects of mating with relatives on gestation time in Poeciliids: Some studies show that it increases (e.g., Pitcher et al. 2008), while others show no difference in gestation time (e.g., Ala-Honkola et al. 2009). Further experiments measuring egg fertilization following artificial insemination might yield more information about the mechanism, if any, by which females reduce the likelihood of inbreeding.

Conclusions

Studies often report reduced reproductive performance of females mating with related males and attribute this to inbreeding depression (i.e., genetic effects). These studies, however, almost always ignore the potential role of post-mating maternal effects in response to the identity of their mating partner. Here, we show a reduction in the number of offspring produced when females mated with a full sibling in the mosquitofish, a species that has limited opportunity to influence this trait via maternal effects. Furthermore, there was no difference between females mated to related or unrelated males in traits that we expected to be influenced by maternal effects (gestation time and whether they breed) or in traits that are unlikely to be affected by maternal affects (offspring birth size and growth). A comparative study measuring inbreeding effects in species that vary in their ability to alter offspring traits via post-mating maternal effects is needed. We suggest that Poeciliids are an ideal group in which to conduct the requisite empirical studies because: (1) closely related species vary substantially in their level of placentation (Pollux et al. 2014), hence ability to adjust provisioning of nutrients to offspring, depending on the relatedness of their mate; (2) the risk of inbreeding seems to have played a role in mate choice in some Poeciliids (e.g., Zajitschek and Brooks 2008) so an adaptive phenotypically plastic maternal response based on relatedness to males with whom they mate is plausible.

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Data Accessibility

Data will be deposited in Dryad upon acceptance.

Conflict of Interest

None declared.

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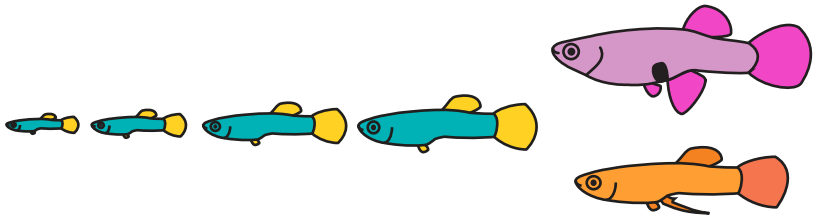
Chapter 3

Inbreeding depression does not increase after exposure to a stressful environment: a test using compensatory growth

BMC Evolutionary Biology 16(1): 68



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RESEARCH ARTICLE Open Access

Inbreeding depression does not increase after exposure to a stressful environment: a test using compensatory growth

Regina Vega-Trejo*, Megan L. Head and Michael D. Jennions

Abstract

Background: Inbreeding is often associated with a decrease in offspring fitness ('inbreeding depression'). Moreover, it is generally assumed that the negative effects of inbreeding are exacerbated in stressful environments. This G × E interaction has been explored in many taxa under different environmental conditions. These studies usually manipulate environmental conditions either in adulthood or throughout an individual's entire life. Far fewer studies have tested how stressful environments only experienced during development subsequently influence the effects of inbreeding on adult traits.

Results: We experimentally manipulated the diet (control versus low food) of inbred and outbred juvenile Eastern mosquitofish (*Gambusia holbrooki*) for three weeks (days 7-28) to test whether experiencing a presumably stressful environment early in life influences their subsequent growth and adult phenotypes. The control diet was a standard laboratory food regime, while fish on the low food diet received less than 25 % of this amount of food. Unexpectedly, despite a large sample size (237 families, 908 offspring) and a quantified 23 % reduction in genome-wide heterozygosity in inbred offspring from matings between full-siblings ($f = 0.25$), neither inbreeding nor its interaction with early diet affected growth trajectories, juvenile survival or adult size. Individuals did not mitigate a poor start in life by showing 'compensatory growth' (i.e. faster growth once the low food treatment ended), but they showed 'catch-up growth' by delaying maturation. There was, however, no effect of inbreeding on the extent of catch-up growth.

Conclusions: There were no detectable effects of inbreeding on growth or adult size, even on a low food diet that should elevate inbreeding depression. Thus, the long-term costs of inbreeding due to lower male reproductive success we have shown in another study appear to be unrelated to inbreeding depression for adult male size or the growth rates that are reported in the current study.

Keywords: Fitness, Food stress, Catch-up growth, Growth rates, Mosquitofish

Background

Mating with relatives occurs commonly in small populations and can result in a decline in offspring performance (ideally measured as fitness) known as inbreeding depression [1]. Inbreeding depression typically has important consequences for variation in lifetime fitness and juvenile development both within and among populations [1, 2]. Due to an increase in homozygosity, inbreeding can reduce performance by either decreasing the frequency of heterozygotes (overdominance) or unmasking deleterious recessive alleles (partial dominance; [3]). Regardless of the mechanism by which inbreeding depression arises, it is usually more readily detected in traits that are linked with fitness (e.g. key life history traits such as growth rates, size at adulthood, and juvenile survival; [4–7]). This is because strong directional selection promotes fixation of advantageous genes, which means that traits linked with fitness have a higher proportion of dominance relative to additive genetic variance [8–10]. Many studies show that inbreeding affects individual traits (e.g. life history, morphology, physiology, and behaviour; [11, 12]). Even so, our understanding of what factors cause variation in the extent to which inbreeding has deleterious effects, and why some traits are affected but not others, remains limited.

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The extent of inbreeding depression may be affected by the environment an individual experiences [13]. Stressful environments (i.e. environments that reduce fitness relative to other environments; [14]) are generally expected to exacerbate the effects of inbreeding [1, 14, 15]. However, over a broad range of taxa and conditions, studies looking at the interaction between inbreeding and stressful conditions have yielded inconsistent results [16–18]. Different species, populations, inbred lines, sexes, and families are highly variable in their response to inbreeding and different types of stress [18–20]. An extensive review by Armbruster et al. [14] found that inbreeding depression increased by 69 % on average in stressful environments, but increased significantly in fewer than half the studies. More recently, a meta-analysis has suggested that the effect of the environment on inbreeding scales linearly with the magnitude of the stress imposed [16]. Thus it appears that the level and type of stress experienced play some part in explaining variation in the severity of inbreeding depression.

A further explanation for the inconsistent effects that stressful environments have on inbreeding depression is that it depends on the developmental or life history stage at which stress is experienced [21–23]. However, most studies look at how stressful environments experienced during adulthood or throughout an organism's life influence the effects of inbreeding [14, 16]. Relatively few studies investigate how stressful environments experienced during particular life stages and, more specifically, during early-life affect the subsequent performance of inbred and outbred individuals [13, 24]. Only six studies in a major review by Fox and Reed [16] explored the interaction between inbreeding and an environmental stress that was restricted to early in life.

A restricted diet during development has the potential to reduce adult body size and consequently lower fecundity, increase predation, and reduce mating success, among other costs [25–29]. Given the potential fitness costs of small adult body size, animals often respond to periods of diet restriction during their juvenile growth phase by increasing growth rates once their diet returns to normal ('compensatory growth') or by delaying maturity until they reach a normal size ('catch-up growth'; meta-analysis: [30]). However, these responses often incur costs such as increased predation risk, changes in locomotor performance, and a reduced lifespan (see [28] for a review). The lack of studies that explore the relationship between inbreeding and a dietary stress early in life is unexpected given the burgeoning interest in 'compensatory growth' to make up for a 'poor start' in life (reviews: [29, 30]) with putative long term costs of elevated 'catch-up' growth [31, 32]. To date, there are surprisingly few experimental studies documenting levels of inbreeding depression that use restricted food availability early in life as an environmental stress and measure its effects on growth and any carry-over effects on size at maturity or other adult traits (but see [4, 33–36]). It is reasonable to assume that the ability to respond to a restricted diet during early development will depend on genotype (e.g. level of heterozygosity, additive genetic variation for fitness; [4, 28]), including the decline in heterozygosity that arises with inbreeding.

Here, we manipulate the amount of food given to experimentally create inbred (F_1 offspring of matings between full siblings, $f = 0.25$) and outbred (F_1 offspring of unrelated parents) juvenile Eastern mosquitofish (*Gambusia holbrooki*). Fish in the control treatment received the standard laboratory diet, while those on a low food treatment received less than 25 % of this amount of food for a 21-day period during early development (days 7-28 after birth) before returning to the control diet. We used data from over 3000 SNPs to confirm that inbreeding reduced genome-wide heterozygosity. We then quantified the interaction between inbreeding and experiencing a presumably more stressful rearing environment. Specifically, we aim to test whether diet restriction during early development differentially influences subsequent growth trajectories and adult phenotype depending on whether an individual is inbred or outbred.

Previous work has shown that female, but not male, *G. holbrooki* show compensatory growth when assigned to our low food treatment, and that both sexes exhibit catch-up growth, albeit with a proportionately longer delay in maturation time for males than females [37]. In addition, we have shown that males reared on the low food treatment are less attractive to females [38]. This suggests that they are less fit so, by definition (sensu [14]), the low food treatment is 'stressful'.

To date there have been almost no studies experimentally manipulating inbreeding in *G. holbrooki* (but see [39]). More generally, however, there is good evidence that inbreeding lowers a range of performance measures in another poeciliid fish, the guppy (e.g. fecundity [40], male reproductive performance [41], sperm number [42, 43], clutch size, and survival [44, 45]). We did, however, use a subset of the current data [46] to show that there is no effect of inbreeding on size at birth and growth over the first seven days in *G. holbrooki*. There is, however, a decline in brood size suggestive of inbreeding elevating embryo mortality. More importantly, we have recently shown that the inbred sons of full-siblings gain a lower share of paternity when they compete with outbred males (Vega-Trejo, R, Head ML, Keogh SJ, Jennions MD unpublished observations). Finally, Kruuk et al. [47] recently reported consistent variation among families in their growth rate on control and low food diets. Given inbreeding generally lowers performance it seems worthwhile to test whether the more 'extreme' genotypes created by inbreeding extend the genetic variation beyond that naturally occurring which might then explain some of the variation in growth patterns.

Given these previous studies we predict that:

(a) Inbred fish will generally have slower growth rates, take longer to mature, and be smaller at adulthood than outbred fish (i.e. inbreeding depression for growth and size).
(b) Inbreeding depression will be greater when fish are placed on a restricted diet as juveniles (i.e. a G × E interaction between inbreeding and diet).
(c) Inbred fish will show weaker compensatory and/or catch-up growth than outbred individuals (i.e. this is the mechanism generating the G × E interaction).

Results

Inbreeding and heterozygosity

We confirmed that there is sufficient genetic variation in our study population for a full-sibling mating to have a readily detectable effect on offspring heterozygosity. Based on data from over 3000 SNP loci, we found that a brother-sister mating led to a significant decline in offspring heterozygosity ($F_{(1,120)} = 215.1$, $P < 0.001$). The mean heterozygosity of inbred fish was 23.2 % less than that of outbred fish (very close to a 25 % decline, which is the expected reduction in heterozygosity due to a full-sib mating in an outbred population). The proportion of loci that were heterozygous was 0.239 ± 0.003 in inbred males ($n = 62$) and 0.311 ± 0.004 in outbred males ($n = 62$). Hereafter we therefore use inbred versus outbred status in our analysis.

Is there an effect of inbreeding on mosquitofish?

Contrary to our predictions, we did not find any evidence of inbreeding depression. This was the case in both the control environment, and in the stressful low food environment that led to almost zero growth over the three-week period in which food was restricted (see below). We have previously reported the effects of inbreeding on birth size and growth to 7 days using a subset of the current data [46]. With the current larger dataset we still found no difference in size at birth, or size at one week of age (before the diet treatment was imposed) between inbred and outbred fish (see also [46]). We also found no significant effect of inbreeding on growth rates, adult size, age at maturity, survival until adulthood, or the sex ratio at maturity (Tables 1 and 2).

Is inbreeding depression exacerbated under a stressful environment?

Contrary to our predictions, we did not find any evidence of an interaction between inbreeding and the diet treatment for any of the nine traits measured (Table 1). There is therefore no evidence that inbreeding depression for these traits is elevated after individuals are exposed to the more stressful low food environment.

Does diet affect growth rate in mosquitofish?

Note, when testing for an effect of diet on growth rate we always included inbreeding status in the model. Prior to imposing the diets, we found a sex difference in growth from birth to one week of age due to females growing significantly faster (Table 1). Given that control diet fish were fed *ad libitum* with *A. nauplii* twice a day throughout the experiment and low food diet had their food restricted from 7 to 28 days of age when they were fed 3 mg of *A. nauplii* once every other day, we found a significant difference between fish on the control and low food diet in the mean growth rate from day 7 to day 28. As expected, the low food diet almost totally suppressed growth, resulting in far smaller fish by day 28. Females still grew significantly faster than males when fish were on the control diet, but not when on the low food diet, presumably because there was so little growth by either sex (Tables 1 and 2).

When fish on the low food diet were returned to the same diet as that of control fish, they showed a significant increase in growth from day 28 to 49 compared to control fish. This was, however, due to their smaller size at the beginning of this period. We did not find any evidence of initial compensatory growth when comparing growth from a comparable starting size (Fig. 1). Although fish on each diet had a similar starting size (that is, growth from day 7 – 28 for control diet and growth from day 28-49 for low food diet fish; Table 2), those on the low food diet actually showed significantly slower growth immediately after returning to a normal diet. In general, after day 28 (the end of the low food diet), females grew significantly faster than males regardless of diet treatment. We did not find any evidence for overall compensatory growth; growth to sexual maturity was not affected by diet nor did it differ between the sexes.

We found some evidence for catch-up growth in mosquitofish. Fish exposed to the low food diet took significantly longer to mature and although statistically they were significantly smaller at maturity, they were still very similar in size to control fish (see below). Females matured at a significantly larger size than males when on the control diet, but not when they were on the low food diet (i.e. sex × diet interaction, GLMM then run separately for each food treatment: Control diet $P = 0.003$, Low food diet $P = 0.687$, Table 1). Females took significantly longer to reach maturity than did males. Males on the low food diet matured on average 20 days later than those on the control diet, while females on the low food diet took 28 days longer to mature than those on the control diet. We did not find any statistically significant sex by diet interactions for time to, or size at maturity. On average, low diet treatment males matured at 98.5 % of the size of the average control diet male and females matured at 96.3 % of the size of the average control diet female (Tables 1 and 2, Fig. 2).

Table 1 Results from mixed models with chi squares (χ^2) values for significance tests of estimated parameters for inbreeding and food treatment

Response variable	*N*	Predictor	Estimate	SE	χ^2	*P*
Length at birth [ln(mm)]	1221	Intercept	0.869	0.002	47498.302	**<0.001**
		Inbreeding (inbred)	3.52×10^{-4}	2.64×10^{-3}	0.046	0.892
Growth day 0 – day 7 (ln[mm]/day)	OM: 234IM: 241OF: 233IF: 200	Intercept	0.057	5.2×10^{-4}	11701.432	**<0.001**
		Inbreeding (outbred)	6.2×10^{-4}	4.0×10^{-4}	2.355	0.125
		Sex (male)	-5.9×10^{-4}	2.5×10^{-4}	5.456	**0.020**
		Inbreeding × Sex	3.1×10^{-4}	2.5×10^{-4}	1.510	0.220
Growth day 7 – day 28 (ln[mm]/day)	OM: 234 IM: 241 OF: 233 IF: 200	Intercept	1.4×10^{-2}	1.15×10^{-4}	16580.458	**<0.001**
		Inbreeding (outbred)	7.6×10^{-5}	9.6×10^{-5}	0.616	0.432
		Diet (control)	1.1×10^{-2}	8.1×10^{-5}	21098.343	**<0.001**
		Sex (male)	-2.4×10^{-4}	8.2×10^{-5}	8.684	**0.003**
		Inbreeding × Diet	-8.7×10^{-5}	8.1×10^{-5}	1.156	0.282
		Diet × Sex	-4.0×10^{-4}	8.3×10^{-5}	23.766	**<0.001**
		Inbreeding × Sex	-5.5×10^{-5}	8.3×10^{-5}	0.447	0.503
		Inbreeding × Diet × Sex	8.9×10^{-5}	8.3×10^{-5}	1.143	0.284
Growth day 28 – day 49 (ln[mm]/day)	OM: 234 IM: 241 OF: 233 IF: 200	Intercept	1.3×10^{-2}	2.2×10^{-4}	3666.595	**<0.001**
		Inbreeding (outbred)	7.2×10^{-5}	1.7×10^{-4}	0.177	0.673
		Diet (control)	-7.6×10^{-3}	9.1×10^{-5}	6939.440	**<0.001**
		Sex (male)	-3.8×10^{-4}	9.6×10^{-5}	16.263	**<0.001**
		Inbreeding × Diet	2.8×10^{-5}	9.1×10^{-5}	0.097	0.756
		Diet × Sex	1.8×10^{-4}	9.6×10^{-5}	3.510	0.061
		Inbreeding × Sex	-4.6×10^{-5}	9.6×10^{-5}	0.229	0.632
		Inbreeding × Diet × Sex	-6.9×10^{-5}	9.6×10^{-5}	0.514	0.474
Initial compensatory growth—Growth control diet (7-28) vs low food diet (28-49) (ln[mm]/day)	OM: 234 IM: 241 OF: 233 IF: 200	Intercept	2.4×10^{-2}	1.8×10^{-4}	16803.581	**<0.001**
		Inbreeding (outbred)	1.3×10^{-5}	1.4×10^{-4}	0.009	0.9262
		Diet (control)	2.5×10^{-3}	1.0×10^{-4}	600.251	**<0.001**
		Sex (male)	-5.4×10^{-4}	1.0×10^{-4}	26.422	**<0.001**
		Inbreeding × Diet	-8.9×10^{-6}	1.0×10^{-4}	0.008	0.9305
		Diet × Sex	-3.7×10^{-5}	1.0×10^{-4}	0.126	0.7227
		Inbreeding × Sex	6.4×10^{-5}	1.0×10^{-4}	0.368	0.544
		Inbreeding × Diet × Sex	1.7×10^{-5}	1.0×10^{-4}	0.028	0.868
Overall compensatory growth—Growth from 7 (control diet) or 28 (low food diet) to sexual maturity (ln[mm]/day)	OM: 233 IM: 241 OF: 233 IF: 198	Intercept	0.041	0.001	1542.2322	**<0.001**

Table 1 Results from mixed models with chi squares (χ^2) values for significance tests of estimated parameters for inbreeding and food treatment *(Continued)*

		Inbreeding (outbred)	8.9×10^{-4}	6.2×10^{-4}	2.036	0.154
		Diet (control)	-1.6×10^{-4}	5.3×10^{-4}	0.087	0.768
		Sex (male)	-1.5×10^{-4}	5.4×10^{-4}	0.074	0.786
		Inbreeding × Diet	-3.1×10^{-4}	5.3×10^{-4}	0.346	0.556
		Diet × Sex	2.7×10^{-4}	5.5×10^{-4}	0.248	0.619
		Inbreeding × Sex	5.6×10^{-4}	5.5×10^{-4}	1.066	0.302
		Inbreeding × Diet × Sex	3.4×10^{-4}	5.5×10^{-4}	0.524	0.469
Catch-up growth—Length at maturity [ln(mm)]	OM: 233 IM: 241 OF: 233 IF: 199	Intercept	1.364	1.8×10^{-3}	5.3×10^{-5}	**<0.001**
		Inbreeding (outbred)	-1.0×10^{-3}	1.4×10^{-3}	0.484	0.487
		Diet (control)	5.7×10^{-3}	1.2×10^{-3}	21.57	**<0.001**
		Sex (male)	-2.1×10^{-3}	1.2×10^{-3}	2.94	0.086
		Inbreeding × Diet	-4.2×10^{-5}	1.2×10^{-3}	1.2×10^{-3}	0.972
		Diet × Sex	-2.8×10^{-3}	1.2×10^{-3}	5.019	**0.025**
		Inbreeding × Sex	5.5×10^{-5}	1.2×10^{-3}	2.0×10^{-3}	0.964
		Inbreeding × Diet × Sex	8.5×10^{-4}	1.2×10^{-3}	0.460	0.498
Catch-up growth—Age at sexual maturity [ln(days)]	OM: 233 IM: 241 OF: 233 IF: 199	Intercept	4.501	0.023	39313.078	**<0.001**
		Inbreeding (outbred)	-0.016	0.016	1.014	0.314
		Diet (control)	-0.131	0.013	107.673	**<0.001**
		Sex (male)	-0.031	0.012	5.723	**0.017**
		Inbreeding × Diet	0.013	0.012	1.001	0.317
		Diet × Sex	0.009	0.013	0.477	0.489
		Inbreeding × Sex	0.018	0.013	1.979	0.159
		Inbreeding × Diet × Sex	0.005	0.013	0.154	0.694
Survival from day of birth to maturity		Intercept	20.217	177.037	0.013	0.909
		Inbreeding (outbred)	0.064	175.037	0	0.999
		Diet (control)	-0.023	192.792	0	0.999
		Sex (male)	-0.052	180.058	0	0.999
		Inbreeding × Diet	5.144	177.420	8×10^{-4}	0.977
		Diet × Sex	5.172	177.257	9×10^{-4}	0.977
		Inbreeding × Sex	-5.234	177.075	9×10^{-4}	0.976
		Inbreeding × Diet × Sex	-0.027	178.041	0	0.999
Offspring sex ratio (proportion male)		Intercept	-0.096	0.067	2.058	0.151
		Inbreeding (outbred)	0.091	0.067	1.882	0.170
		Diet (control)	-0.032	0.067	0.238	0.626
		Inbreeding × Diet	0.037	0.067	0.303	0.582

Numbers in bold indicate significant values. *OM* outbred males, *IM* inbred males, *OF* outbred females, *IF* inbred females. N varied in the analysis due to individuals not being measured at adulthood or died

Table 2 Means and SE from raw data separated by sex and food treatment

	Outbred		Inbred	
Length at birth (mm)	7.375 (0.017)		7.378 (0.016)	
	Outbred control diet	Inbred control diet	Outbred low food diet	Inbred low food diet
Male growth day 0 – day 7 (ln[mm]/day)	0.058 (0.0007)	0.057 (0.0007)	0.056 (0.0007)	0.055 (0.0008)
Male length at day 7 (mm)	11.101 (0.057)	11.005 (0.062)	10.974 (0.059)	11.017 (0.073)
Male growth day 7 – day 28 (ln[mm]/day)	0.026 (0.0002)	0.026 (0.0002)	0.003 (0.0001)	0.003 (0.0002)
Male length at day 28 (mm)	19.133 (0.090)	18.942 (0.096)	11.725 (0.079)	11.770 (0.092)
Male growth day 28 – day 49 (ln[mm]/day)	0.006 (0.0002)	0.006 (0.0002)	0.021 (0.0003)	0.021 (0.0003)
Male compensatory growth control diet (7-28) vs low food diet (28-49) (ln[mm]/day)	0.026 (0.0002)	0.026 (0.0002)	0.021 (0.0003)	0.021 (0.0003)
Male catch-up growth control diet (7-maturity) vs low food diet (28-maturity) (ln[mm]/day)	0.040 (0.002)	0.040 (0.001)	0.042 (0.002)	0.040 (0.002)
Male length at maturity (mm)	23.243 (0.175)	23.302 (0.173)	22.779 (0.135)	23.047 (0.139)
Male age at sexual maturity (days)	80.570 (3.471)	77.298 (2.817)	97.258 (3.368)	100.479 (3.398)
Female growth day 0 – day 7 (ln[mm]/day)	0.059 (0.0008)	0.058 (0.0008)	0.058 (0.0007)	0.058 (0.0008)
Female length at day 7 (mm)	11.145 (0.065)	11.124 (0.069)	11.162 (0.058)	11.084 (0.059)
Female growth day 7 – day 28 (ln[mm]/day)	0.027 (0.0003)	0.027 (0.0003)	0.003 (0.0002)	0.002 (0.0001)
Female length at day 28 (mm)	19.683 (0.123)	19.707 (0.127)	11.919 (0.078)	11.688 (0.078)
Female growth day 28 – day 49 (ln[mm]/day)	0.007 (0.0002)	0.006 (0.0003)	0.022 (0.0003)	0.022 (0.0003)
Female compensatory growth control diet (7-28) vs low food diet (28-49) (ln[mm]/day)	0.027 (0.0003)	0.027 (0.0003)	0.022 (0.0003)	0.022 (0.0004)
Female catch-up growth control diet (7-maturity) vs low food diet (28-maturity) (ln[mm]/day)	0.043 (0.001)	0.040 (0.001)	0.042 (0.002)	0.040 (0.002)
Female length at maturity (mm)	23.617 (0.203)	23.920 (0.215)	22.857 (0.211)	22.916 (0.246)
Female age at sexual maturity (days)	78.781 (3.964)	83.084 (3.926)	104.193 (4.146)	113.615 (5.096)
Survival	96.20 %	93.19 %	95.58 %	90.95 %
Sex ratio (M:F)	114:114	124:96	120:119	117:104

Finally, neither juvenile survival nor sex ratio at maturation was affected by diet (Table 2).

Discussion

The effects of inbreeding are expected to be exacerbated in stressful environments [14]. We tested this hypothesis by rearing inbred and outbred mosquitofish in two different food treatments (i.e. a stressful environment — low food diet and a non-stressful environment — control diet) and measured their growth rate, size, age at maturity, and their ability to show compensatory growth and catch-up growth. Our results revealed (1) no evidence for inbreeding depression in either the benign or more stressful rearing environments, (2) some evidence for catch-up growth, and (3) no evidence for compensatory growth.

We found no evidence for inbreeding depression for any of the measured traits (i.e. growth rates, adult size, and age at maturity). One reason that is often posited for a lack of inbreeding depression is that the expression of deleterious alleles depends on the environment an animal experiences [14], including the conditions in which animals are raised [48]. For example, previous studies have shown effects on inbreeding in the presence of certain stressors (e.g. chemicals or desiccation), but not others (e.g. heat resistance; [49]). Others have found a modest correlation between the extent of inbreeding depression and the level of dietary stress [50–52]. Our low food diet lead to almost zero growth over a three-week period and is thus comparable to a very harsh natural environment. The fact that we did not find effects of inbreeding depression in either of our experimental treatments, especially given our large sample size ($N = 908$ fry), is thus robust evidence that the traits we measured do not suffer inbreeding depression in *Gambusia holbrooki* under the stressful conditions the fish experienced in this experiment (i.e. three weeks with insufficient food for juvenile growth). We have previously shown [38] that this diet reduces male attractiveness and is therefore, by definition, stressful (see [14]).

The presence and magnitude of inbreeding depression may differ depending on which life stages and/or traits are measured [53]. For example, some studies show no effect of inbreeding depression on body size, but do show an effect on time to development [7]. The traits we measured (i.e. growth, time to maturation, survival)

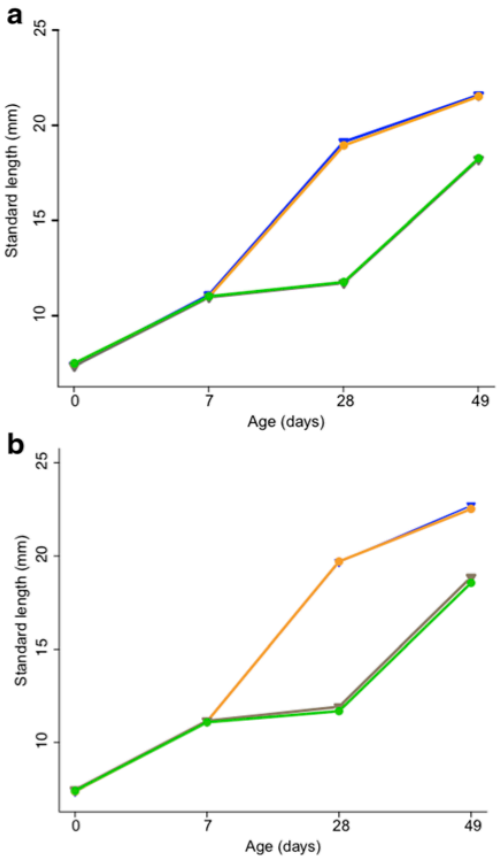

Fig. 1 Mean growth trajectories of fish separated by inbreeding and diet. Data shown for growth periods prior to sexual maturity for (**a**) females, (**b**) males. Blue triangles = outbred control diet, brown triangles = outbred low food diet, orange circles = inbred control diet, green circles = low food diet

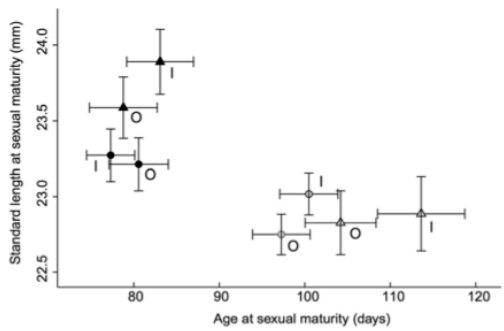

Fig. 2 Mean age and length at sexual maturity and 95 % confidence interval for fish separated by inbreeding, diet, and sex. O = outbred, I = inbred, triangles = females, circles = males, black = control diet, grey = low food diet. Outbred control males $N = 114$, Outbred control females $N = 114$, Outbred low food diet males $N = 119$, Outbred low food diet females $N = 119$, Inbred control males $N = 124$, Inbred control females $N = 95$, Inbred low food diet males $N = 117$, Inbred low food diet females $N = 104$

are major life-history traits with large effects on fitness in many species [25, 54] that are therefore expected to be condition-dependent [55]. These traits should be particularly prone to inbreeding depression because condition is assumed to be affected by multiple loci across the genome [10], so this result was somewhat surprising. One explanation for a lack of inbreeding effect is that maternal and family effects on fitness might overshadow effects associated with inbreeding [39, 56] due to high variance among families [57]. We can dismiss this explanation, however, as we explicitly controlled for sire, dam, and family effects. Another explanation for a lack of inbreeding depression for the traits we measured is that mosquitofish have purged deleterious alleles for metabolic responses to low food availability as a result of periodic population bottlenecks [58, 59]. In support of this, previous studies looking at the effects of inbreeding depression on population size and population growth rate under two different salinities in mosquitofish did not find evidence for inbreeding depression [39]. However, in our population we have directly shown that lower heterozygosity in males (natural rather than experimental in origin) leads to significantly lower reproductive success (Head ML, Kahn AT, Keogh SJ, Jennions MD unpublished observations), suggesting that inbreeding *does* reduce fitness, but not because of its effects on adult size or growth rates.

We did not find any evidence of compensatory growth in our study. Fish in the stressful low food environment did not show faster growth rates after food restriction early in life compared to fish on the control diet. This result, contrasts with that of Livingston et al. [37] who found partial compensatory growth for females, but it agrees with their findings for males. Both studies used the same diet manipulation so the reasons for the difference are unclear. However, our findings are in accordance with the wider trend that fish generally show little evidence for compensatory growth compared to other taxa [30]. One reason that has been posited for this taxonomic difference is that ectotherms have indeterminate growth and are under less pressure to rapidly achieve a large final size than taxa with determinate growth. However, the evidence from mosquitofish does not support this explanation. Male mosquitofish have determinate growth but do not show compensatory growth (this study and [37]), while females have indeterminate growth but there is some evidence for compensatory growth ([37], but not our study). If we assume selection for large body size is comparable across the sexes (although this might not be the case in Poeciliids where

smaller males could have a mating advantage: see [60] and Head ML, Kahn AT, Keogh SJ, Jennions MD unpublished observations) we would expect to see compensatory growth in males, but not females, if an explanation based on determinate versus indeterminate growth is correct.

Although we did not observe compensatory growth in response to food deprivation, fish in the low food diet did mature at a very similar (albeit statistically significantly smaller) size to those on the control diet because they delayed their maturation (i.e. 'catch-up growth' sensu [30]). Similar results have been found for another poeciliid fish the guppy (*Poecilia reticulata*) [61, 62]. In these studies, guppies showed a reduction in growth rate, an increase in age at maturity, and a decrease in size at maturity after a period of reduced food availability. Delaying maturation to achieve a larger adult size may be physiologically less costly than increasing growth rate [63], but it could still reduce lifetime reproductive success if it leads to less time in the breeding pool [64]. The relative magnitude of these two costs could be important in determining whether species compensate for restricted growth during development by increasing their subsequent growth or by delaying maturation.

Conclusions

There was no interaction between inbreeding and diet restriction during development on juvenile survival, growth or size, and age at maturity. This indicates that these traits do not suffer from inbreeding depression, even after individuals are exposed to a seemingly stressful low food environment (see [38]). It implies that how mosquitofish respond to a restricted diet during early development does not depend on phenotypic quality (assuming inbred individuals are, at least for some traits, inferior due to their lower heterozygosity). Of course, our results do not rule out that inbreeding depression occurs in *G. holbrooki*, nor do they exclude a G × E interaction between inbreeding and rearing environment. Previous studies highlight that it is important to look at the effects of inbreeding over all life stages and for multiple traits [13]. Looking at only single life stages or a limited set of traits may under- or overestimate the effects of inbreeding because it does not take into account potential trade-offs between life stages or traits [11, 13, 65]. For example, in mosquitofish, males that have a poor start in life (i.e. reared on a restricted diet) are less attractive to females than those reared on a control diet in simple two-choice mate association tests [38]. This illustrates the potential for hidden long-term costs of a stressful environment. Furthermore, we reared fish individually (to reduce variation), but this eliminates any potential for reduced social competitiveness to affect growth and adult size. Perhaps most importantly, in a companion study we tested how the inbreeding status and diet treatment of males affect their ability to gain paternity when they compete for females in a socially competitive environment (Vega-Trejo, R, Head ML, Keogh SJ, Jennions MD unpublished observations). We found that inbred males are significantly less successful, but that there is no effect of diet, nor any interaction between diet and inbreeding on male reproductive success. This suggests that inbreeding *does* ultimately reduce fitness and perhaps overrides the effect seen in attractiveness due to diet [38]. The current study indicates, however, that this is not because inbreeding affects adult size or growth rates. The proximate basis of inbreeding depression in male *G. holbrooki* therefore remains to be determined. One possibility that we are currently testing is that inbreeding lowers sperm competitiveness.

Methods

Study system

Mosquitofish (*Gambusia holbrooki*) are small Poeciliid fish endemic to North America and introduced worldwide [66]. They are non-migratory and are often resident in relatively small bodies of water such as ponds and streams [67]. This makes it likely that inbreeding occurs naturally in situations where a few fish become isolated in a small area.

Origin and maintenance of fish

Our laboratory stock of mosquitofish derives from 151 wild-caught gravid females (females mate multiply so broods have multiple sires) collected in Canberra, Australia in February and March 2013. This work was conducted under the ethic approval that was granted by ANU animal ethics protocol A2011/64. Collection permits were not required for this study as *G. holbrooki* are a pest species in Australia. F_1 generation offspring were kept in single sex tanks under a 14:10 h photoperiod at 28 °C and fed *ad libitum* with *Artemia nauplii* and commercial flakes. Females were reared to adulthood and separated before sexual maturity to ensure virginity.

Experimental design

The design to create inbred and outbred fish is fully described in Vega-Trejo et al. [46]. In brief, we set up 150 unique breeding pairs that were randomly created from F_1 individuals (described above, avoiding any pairing of fish with the same mother). From these pairings we obtained 58 outbred F_2 full-sib families with sufficient numbers of both sexes to be used in our experimental design. The design required two F_2 families per block to create both inbred and outbred offspring (described below). We established 29 experimental blocks.

Inbred versus outbred fish

We used a fully balanced block design that involved mating individuals from two families (e.g. A and B). Brothers

and sisters from full sibling families were paired to create inbred offspring (AA, BB) and outbred offspring with reciprocal crosses for each cross-type (BA, AB; Fig. 3). Males and females were placed together for 1 week to allow mating. Females were then placed in individual 1 L tanks and checked twice daily for babies over a six-week period. Those that had not given birth were re-introduced to the male for another 7 days to increase the number of offspring produced. We recorded gestation time, female standard length (SL = snout tip to base of caudal fin) and the number of offspring produced [46]. To measure female size, fish were anaesthetized by submersion in ice-cold water for a few seconds to reduce movement and then photographed alongside a microscopic ruler (0.1 mm gradation). We also recorded the size of offspring within 18 h of being born using images obtained after placing live fish into a square container (27 wide × 27 mm long × 22 high) containing water to a depth of 1 mm. Measurements were made using *Image J* software [68]. These, and all subsequent, size measures were made blind to treatment type (see [69]).

Diet

We raised a maximum of 10 fry from each cross-type, each reared individually in separate 1 L tanks. All fish were fed *ad libitum* with *A. nauplii* twice a day for seven days and then photographed for later measurements (as described above). Each fish was then randomly assigned to the control or low food diet at one week of age. Control diet fish continued being fed *ad libitum* with *A. nauplii* twice a day until the end of the experiment ($N = 472$). Fish in the low food diet had their food restricted from 7 to 28 days of age (i.e. experienced limited food availability for 21 days) when they were fed 3 mg of *A. nauplii* once every

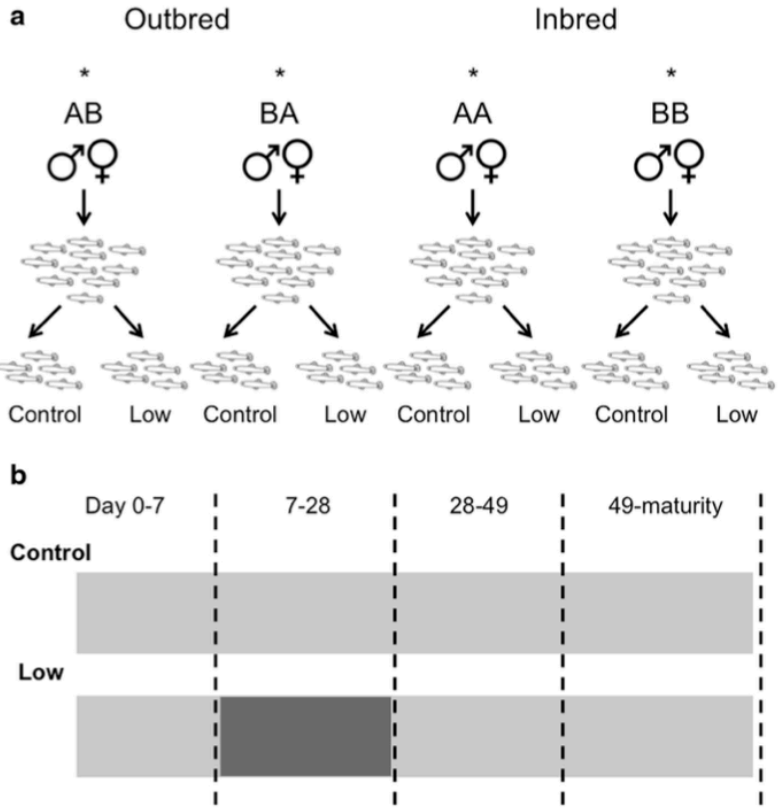

Fig. 3 Schematic of experimental design. **a** Shows block design used to create outbred and inbred fish. For each block we set up 1-4 females per cross-type. Within each block the same potential number of females contributed to each cross-type. A single male contributed to each cross-type so that, within each block, the offspring of each cross-type were either full or paternal half-siblings. We ended up with 604 inbred offspring from 109 mothers and 54 fathers, and 617 outbred offspring from 128 mothers and 55 fathers. Offspring from each cross-type were evenly distributed across food treatments. * indicate matings, **b**) shows feeding regime for each diet treatment. Light shade indicates Ad lib food was given twice a day, dark shade indicates 3 mg of food every other day. Dashed lines indicate points at which measurements were taken

other day (less than 25 % of the amount of food; $N = 492$). From day 28 onward their diet was returned to the same level as that of control diet fish (Fig. 3). This low food diet minimises growth (see diet effect in Table 1, Fig. 1), but did not increase mortality (see [37]).

Size measurements

All fish were photographed (as for females above) on day 28 (end of low food diet) and again on day 49. Thereafter, fish were inspected three times per week to determine the time to maturity and photographed to obtain their SL once mature. Females were considered mature when yellow spots were evident in the abdomen, indicating yolked eggs [70]. Males were considered mature when their gonopodium (intromittent organ modified from the anal fin) was translucent, with a spine visible at the tip [70, 71]. All inspections for maturity were made blind to treatment. Unexpectedly (see [37]) some, mainly control fish ($N = 133$) matured before day 49 (68 outbred and 51 inbred on control diet; 8 outbred and 6 inbred on low diet). In our analyses we treat these fish are though they matured on day 49. In further sensitivity analyses we alternatively gave control diet individuals lower ages at maturity (between 28 and 49 days). This did not qualitatively alter our results, nor did analysing the effect of inbreeding based only on fish on the low diet treatment (results are not presented, but data is available in Dryad).

Inbreeding and heterozygosity

If we treat the source population as a baseline of outbred individuals then $f = 0.25$ for the offspring of brother-sister matings.

We used RAD-tag to detect SNPS that provided us with data of genome wide heterozygosity based on 3045 SNPs from a subsample of 122 males (see Additional file 1 for full methods). We then quantified the proportion of loci per male that were heterozygous, and tested whether the mean level of heterozygosity differed between inbred and outbred males.

Statistical analysis

Diet & inbreeding effects

We analysed the fixed effect of diet, inbreeding (inbred versus outbred), sex, and all possible two-way and three-way interactions using generalised linear mixed models (GLMM) in *R 3.0.2* software [72] with separate models for each response variable. We ran models for *size at birth, growth rates, size at maturity*, and *age at maturity* using a Gaussian error distribution. We also ran a model for *age at maturity* with a negative binomial distribution of the error due to the fairly high number of fish classified as maturing on day 49. Each model was fitted using the *lme4* package in *R 3.0.2* software with block, maternal identity, and sire identity as random factors. All size measurements were log transformed. All parameters estimated were tested for significance using Anova with Type III Wald chi-square tests. Model simplification (i.e. removing non-significant interaction terms) did not change our results. Figures are presented using raw data rather than model predictions unless otherwise indicated. We have previously reported the effects of inbreeding on birth size and growth to 7 days using a subset of the current data ([46]; the current data set includes offspring produced more than six weeks after initial pairing of fish).

Compensatory growth

There was no initial size difference at birth between inbred and outbred fish (see Results). Additionally, we tested whether inbreeding and/or sex affected growth to day 7 (i.e. the beginning of the diet treatment). Growth was always quantified as the instantaneous rate of growth, $G = \ln(L_{t1}/L_{t0}) / t$, where L refers to the length (SL) at t_n age and t is time (day) of measurement. There was no difference in initial growth to day 7 between inbred and outbred fish (see Results). The fish assigned to the four categories (inbreeding × diet) were therefore the same mean size at the start of the diet treatment.

We tested for an effect of diet on growth while the treatment was applied by comparing the growth of control and low food diet fish between days 7 and 28. We then tested for an early compensatory growth response of low food diet fish by comparing growth when returned to the control diet. To account for a potential effect of a difference in size at the start of the relevant growth period (i.e. because growth slows with absolute size), we compared growth from days 7 – 28 for the control diet fish [$\ln(L_{day\ 28} / L_{day7}) / 21$] and days 28-49 for the low food diet fish [$\ln(L_{day\ 49} / L_{day28}) / 21$] because the mean size of fish in the two groups was very similar at the start of the respective growth periods (mean control diet fish day 7: 11.07 ± 0.03, mean low food diet fish day 28: 11.76 ± 0.04). Then we tested for an overall effect of compensatory growth by testing for a difference in the instantaneous growth rate for each fish from an age giving a comparable initial body size (day 7 for control diet fish, day 28 for low food diet fish) to maturation. The duration of this period varied among individuals within and among treatments due to the time taken to reach maturity. Finally, we tested for catch-up growth evidenced by differences in length and age at maturity.

We also tested for any effect of diet, inbreeding or sex on survival and the offspring sex ratio using models with a binomial distribution of the error. These models used only fish that reached maturity.

Ethics

This work was conducted under the ANU animal ethics protocol, granted by animal use permit: ANU AEEC animal

ethics protocol A2011/64. Collection permits were not required for this study as *G. holbrooki* are a pest species in Australia.

Consent to publish
Not applicable.

Availability of data and materials
Data is deposited in Dryad: doi:10.5061/dryad.mb2gb.

Additional file

Additional file 1: Provides the methods used to obtain data of genome wide heterozygosity. (DOCX 141 kb)

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RV-T participated in the design of the study, performed the laboratory work, performed the statistical analysis and drafted the manuscript. MLH participated in the design of the study, assisted in laboratory work, assisted in statistical analysis and helped to draft the manuscript. MDJ participated in the design of the study, assisted in statistical analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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Additional file 1

To determine heterozygosity for the fish in our experiment we took tissue samples from a subsample of males (n= 122). DNA was extracted from the tail muscle/caudal fin using Qiagen DNeasy Blood and Tissue Kits following the manufacturer's instructions. After extraction DNA samples were sent to the commercial genotyping service Diversity Arrays. This company has developed a widely used technique called DArTseq™. DArTseq™ represents a combination of DArT complexity reduction methods and next generation sequencing platforms [1-4]. It is a new implementation of sequencing complexity reduced representations [5] and more recent applications of this concept on next generation sequencing platforms [6, 7]. The technology is optimized for each organism by selecting the most appropriate complexity reduction method based on both the size of the representation and the fraction of a genome selected for assays. Four methods of complexity reduction were tested in *Gambusia* (data not presented) and the PstI-HpaII method was selected. DNA samples were processed in digestion/ligation reactions principally as per [3] but replacing a single PstI-compatible adaptor with two different adaptors corresponding to two different Restriction Enzyme (RE) overhangs. The PstI-compatible adapter was designed to include Illumina flowcell attachment sequence sequencing primer sequence and "staggered" varying length barcode region similar to the sequence reported by [7]. Reverse adapter contained flowcell attachment region and HpaII-compatible overhang sequence. Only "mixed fragments" (PstI-HpaII) were effectively amplified in 30 rounds of PCR using the following reaction conditions: 1. 94 C for 1 min; 2. 30 cycles of 94 C for 20 sec 58 C for 30 sec 72 C for 45 sec; 3. 72 C for 7 min. After PCR equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2500. The sequencing (single read) was run for 77 cycles.

Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline the fastq files were processed to filter away poor quality sequences applying more stringent selection criteria to the barcode region than the rest of the sequence. In that way, the assignments of the sequences to specific samples carried in the "barcode split" step are very reliable. Approximately 2500000 (+/-

7%) sequences per barcode/sample were used in marker calling in routine DArTseq assay but we applied a more cost-effective version of the assay using half of the normal tag number (average of 1.3 million per sample). Finally, identical sequences were collapsed into "fastqcall files". These files were used in the secondary pipeline for DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation) calling algorithms (DArTsoft14). This clean-up process resulted in a comprehensive data set of approximately 3045 SNPs with an average call rate of 97.7% and a reproducibility rate of 99.3%.

Heterozygosity

We estimated heterozygosity by using the number of markers that were scored as heterozygous divided by the total number of successfully classified markers for that fish.

Supplementary References

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Chapter 4

Are sexually selected traits affected by a poor environment early in life?

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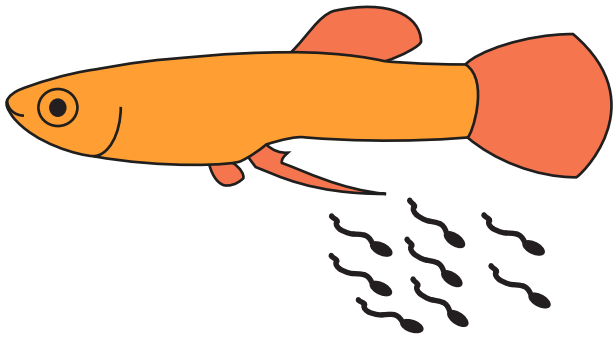

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RESEARCH ARTICLE Open Access

Are sexually selected traits affected by a poor environment early in life?

Regina Vega-Trejo[1*], Michael D. Jennions[1,2] and Megan L. Head[1]

Abstract

Background: Challenging conditions experienced early in life, such as a restricted diet, can detrimentally affect key life-history traits. Individuals can reduce these costs by delaying their sexual maturation, albeit at the price of the later onset of breeding, to eventually reach the same adult size as individuals that grow up in a benevolent environment. Delayed maturation can, however, still lead to other detrimental morphological and physiological changes that become apparent later in adulthood (e.g. shorter lifespan, faster senescence). In general, research focuses on the naturally selected costs of a poor early diet. In mosquitofish (*Gambusia holbrooki*), males with limited food intake early in life delay maturation to reach a similar adult body size to their well-fed counterparts ('catch-up growth'). Here we tested whether a poor early diet is costly due to the reduced expression of sexually selected male characters, namely genital size and ejaculate traits.

Results: We found that a male's diet early in life significantly influenced his sperm reserves and sperm replenishment rate. Shortly after maturation males with a restricted early diet had significantly lower sperm reserves and slower replenishment rates than control diet males, but this dietary difference was no longer detectable in older males.

Conclusions: Although delaying maturation to reach the same body size as well fed juveniles can ameliorate some costs of a poor start in life, our findings suggest that costs might still arise because of sexual selection against these males. It should be noted, however, that the observed effects are modest (Hedges' $g = 0.20–0.36$), and the assumption that lower sperm production translates into a decline in fitness under sperm competition remains unconfirmed.

Keywords: Age, Catch-up growth, Diet, Mosquitofish, Sperm

Background

Conditions experienced early in life affect life-history trajectories [1, 2]. In particular, lower growth rates due to limited food availability during development tend to reduce adult body size [3]. In some species, however, individuals reduce the potential fitness costs of smaller adult body size. For example, if food again becomes available, they compensate by accelerating their growth (compensatory growth). Alternatively, they delay maturity to attain the same adult size as well-fed individuals (catch-up growth; reviews: [4, 5]). There are usually clear benefits to large adult size, such as increased survival and higher reproductive success (e.g. [6]), but reaching the same size as better fed individuals might generate other costs (e.g. [7]). An obvious cost of catch-up growth is a longer generation time and, if there is seasonal breeding, a shorter reproductive lifespan [8, 9]. More subtle costs arise when elevated or extended growth produces developmental abnormalities that can, for example, increase the risk of predation, decrease immune function, and lower resistance to stressors (e.g. [4, 10–12]). Poor nutrition early in life has been shown to adversely affect adult behaviour [11, 13], locomotor performance [14], functional morphology [15, 16], and key adult life-history traits [17–20]. But even if there are no obvious effects, a poor start in life can still decrease fitness. For example, individuals reared on a restricted diet might be morphologically indistinguishable from those reared on a standard diet, but have shorter telomeres or lower plasma antioxidant levels [21–23], which should reduce their adult lifespan (but see [2]).

A major life history allocation decision is how to invest in naturally and sexually selected traits. In general, however, we know little about how conditions early in life

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affect allocation of resources to adult life history traits ([24], but see: [25]). In particular, far more studies have investigated the effects of early diet on naturally rather than sexually selected traits, (but see: [26–29]). This is surprising because variation in male lifetime reproductive success is often predominantly attributed to differences in mating success (i.e. sexual selection; [30]). Most sexually selected traits are under strong directional selection, and food availability is often a major determinant of their condition-dependent expression. Pre-copulatory sexually selected traits (i.e. those that determine mating success) can be detrimentally affected by poor early nutrition. For example, male eye-span in stalked-eyed flies and song repertoire size in great reed warblers, which are both traits that affect female mate choice, are negatively affected by nutritional stress during development [31, 32]. This reduced investment could reflect a life history trade-off between sexually and naturally selected traits. For example, when early nutrition is poor, males sometimes reduce investment in sexual ornaments to maintain their oxidant defence systems [21, 29]. Similar trade-offs could also affect investment into different sexually selected traits [33, 34]. Male reproductive success usually depends on both mating success and sperm competitiveness (e.g. [35–37]). Given lower resource availability, males might invest differently in traits under pre-copulatory and post-copulatory sexual selection (e.g. [38, 39]). This could shift the relative allocation of resources to sperm competitiveness versus attractive ornaments [40]. For example, greater investment in larger body size or weaponry can result in smaller testes and ejaculates [41, 42].

The condition-dependence of sperm traits has been examined in several species, but this is usually due to short-term effects of manipulating the adult diet (e.g. [43–45]). Fewer studies, especially of vertebrates, have tested how a restricted juvenile diet affects sperm traits (but see: [35, 40, 46]). Male fertilization success is highly dependent on resource allocation to traits that are under post-copulatory sexual selection, especially when sperm competition is intense [47]. In such species, males tend to have relatively larger testes that produce more sperm [48, 49]. Of course, sperm production is not the sole predictor of sperm competitiveness. It can also depend on sperm viability, swimming speed, and even sperm length [50, 51]. Since ejaculates are costly to produce [44, 52], it follows that a poor juvenile diet could negatively affect the number, quality, and rate of sperm production (e.g. [53, 54]).

Here we test whether the juvenile diet of male mosquitofish (*Gambusia holbrooki*), despite having no effect on adult body size, affects two sexually selected adult traits: ejaculate production and genital size. In two earlier studies we showed that males with limited food intake as juveniles reach a similar size to males on a normal diet due to delayed maturation [55, 56]. Additionally, we showed that males with a poor start in life are less attractive to females than those reared on a regular diet [57]. Mosquitofish are poecillid fishes characterised by frequent, coercive mating attempts and intense sexual selection, including sperm competition [54, 58, 59]. Males internally inseminate females using a modified anal fin as an intromittent organ, the 'gonopodium' [60]. Several recent studies have linked greater gonopodium length to increased male reproductive success in *G. holbrooki* ([61, 62] but see [63]). We ask whether males initially raised on a restricted diet incur sexually selected costs, despite catch up growth, due to the production of lower quality ejaculates, or development of a shorter gonopodium.

Methods

Fish were bred as part of a larger study to test how inbreeding and food restriction affect compensatory growth [55]. We found no effects of inbreeding on any of the measured life history variables (growth trajectories and adult size). Here, we are specifically interested in whether early diet restriction influences sexually selected traits so, for clarity, we analyse the data excluding inbreeding from our models. Including inbreeding does not qualitatively alter our results because it had very small, non-significant effects. These are discussed elsewhere (J. Marsh, R. Vega-Trejo, M.L. Head, and M.D. Jennions 'in preparation').

We used mosquitofish descended from females captured in ponds in Canberra, Australia (35°14'27"S, 149° 5'27"E and 35°14'13"S, 149° 5'55"E) in February-March 2013. Full methods are described in Vega-Trejo et al. [64]. In brief, in each experimental block we mated individuals from two families (e.g. A and B in block 1, C and D in block 2 and so on). Brothers and sisters from full sibling families were paired to create inbred (AA, BB) and outbred offspring with reciprocal male–female crosses (AB, BA; i.e. four cross-types). We set up 29 blocks and with one male and one to four full sisters per cross type. The resultant offspring were reared individually in 1 L tanks until maturity ($N = 453$ males) under a 14:10 h photoperiod at 28 °C. Males underwent a diet manipulation for 21 days between days 7 and 28 post-birth. Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice daily (i.e. standard laboratory feeding regime) whereas fish on the restricted diet were fed 3 mg of wet mass *Artemia* nauplii once every other day. We have previously shown that this restricted diet leads to minimal growth without elevating juvenile mortality [56]. Broods were split evenly between the control and restricted diet treatments. Males were considered mature when their gonopodium was translucent with a spine visible at the tip [65, 66]. These changes are associated with spermatogenesis in the testes [60, 66]. We have

previously shown that inbreeding (i.e. mating with full-sibs) reduces the number of offspring at birth, but with no detectable effect on their likelihood of breeding, gestation time, offspring size at birth or growth rate [64]. In addition, there is no difference in juvenile survival between males on the control and the restricted diet (i.e. GLMM ran for food treatment: $P = 0.952$; [55]). We collected body size and sperm data from mature males (range: 2–18 weeks post-maturity). We define 'developmental time' as the number of days that males took to reach maturity, and 'adult age' as the post-maturation age at which sperm was extracted (i.e. total age – developmental time). Developmental time was 78.6 ± 34.3 days for males in the control treatment and 99.4 ± 37.5 for males in the restricted diet treatment. Adult age was 81.0 ± 17.1 days for males on the control diet, and 70.4 ± 21.4 days for males on the restricted diet (mean ± SD).

Sperm traits

We tested 453 males from 192 broods. Sperm was collected on three occasions: on day 0 we stripped virgin males of sperm (see below) to measure their maximum sperm reserves; one day later we stripped males to measure their sperm replenishment rate (i.e. sperm production over 24 h); on day 3 we stripped males to measure sperm velocity. We also calculated the proportion of sperm replenished (= number of sperm at day 1/number of sperm at day 0), which we arcsine-transformed to normalize the error distribution.

Sperm collection

To strip ejaculates, males were anaesthetized in ice-cold water. The male was then placed on a glass slide (coated with 1% polyvinyl alcohol solution (PVA) to prevent sperm bundles sticking to the slide) under a dissecting microscope. His gonopodium was swung forward and we applied gentle pressure to the abdomen to eject all the available sperm. We transferred the ejaculate to an Eppendorf tube with 100–900 μL of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5). The amount of medium varied depending on the amount of ejaculate stripped to obtain accurate sperm counts, which require an intermediate sperm concentration. Sperm remain quiescent in this solution until activated [67]. Sperm counts and velocity measures were taken within 30 min of sperm collection (see [51] for further details). After the procedure each male was returned to his individual tank. Sperm collection was done blind to diet treatment by RVT.

Sperm number

To estimate the number of sperm we vortexed the sperm solution for 1 min and then mixed it repeatedly with a pipette (20–30 times) to break up sperm bundles and distribute the sperm evenly throughout the sample. We placed 3 μl of solution on a 20 micron capillary slide (Leja) and counted the sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100× magnification. We counted five subsamples per sample. We estimated repeatability following Nakagawa and Schielzeth [68] using the *rptR* package in *R 3.0.2* [69]. Repeatability was very high for sperm number (sperm at day 0: $r = 0.85 \pm 0.01$ SE; sperm at day 1: $r = 0.91 \pm 0.006$ SE). The mean of the five subsamples was used for further analyses. The threshold values defining cell detection were predetermined as elongation percentage 15–65, head size 5–15 μm, and the static tail filter was set off. Sperm were counted blind to male treatment.

Sperm velocity

For each ejaculate we analysed three samples. For each sample we collected 3 μL of the diluted sperm (above) and placed this in the centre of a cell of a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA) previously coated with 1% PVA. The sample was then activated with a 3 μL solution of 150 mM KCl and 2 mg ml^{-1} bovine serum albumin [70] and covered with a cover slip. We analysed sperm velocity within 30 s of activation for three subsamples to increase the number of velocity measures. We used an average of 109.3 ± 3.5 SE sperm tracks per ejaculate (minimum 10 sperm tracks/male). We excluded six of 399 available males from the velocity analysis because they had fewer than 10 sperm tracks. We recorded two standard measures of sperm velocity: (1) average path velocity (VAP): the average velocity over a smoothed cell path and (2) curvilinear velocity (VCL): the actual velocity along the trajectory using a CEROS Sperm Tracker. The threshold values defining static cells were predetermined at 20 μm/s for VAP and 15 μm/s for VCL. Repeatability was high for both parameters (VAP: $r = 0.65 \pm 0.02$ SE; VCL: $r = 0.58 \pm 0.03$) and we used the mean of the three subsamples in our analyses. Due to the near perfect correlation between VAP and VCL ($r = 0.961$, $P < 0.001$), as found in most comparable studies (e.g. [71–73]), we only use VAP in our analyses.

Male morphology

All males were measured a week after sperm extraction. Males were anaesthetized by submersion in ice-cold water for a few seconds to reduce movement and then placed on polystyrene with a microscopic ruler (0.1 mm gradation) and photographed. We measured male standard length (SL = snout tip to base of caudal fin) and gonopodium length using *Image J* [74].

Statistical analysis

We removed one of 453 males from the analysis because he had a higher number of sperm on day 1 than day 0 indicating that not all sperm were collected during the first extraction. To analyse the effect of diet treatment on male sexual traits we used generalized linear mixed models (GLMM). We constructed separate models for each of our five response variables: gonopodium length, number of sperm at day 0, number of sperm at day 1 (i.e. replenishment rate), proportion of sperm replenished (arc-sine transformed), and VAP. In each model we included diet as a fixed factor, and male standard length, development time, and adult age as fixed covariates, as well as all two-way interactions with diet. Gonopodium length and body size were log-transformed. Adult age was not included in the model for gonopodium length as there is little post-maturity growth in *G. holbrooki*.

There were significant bivariate correlations between development time and body size ($r = 0.62$, $P < 0.001$) as well as development time and adult age ($r = -0.77$, $P < 0.001$). The former reflects a biological relationship and the latter is due to a logistic constraint (i.e. having to terminate the experiment). Even so, these correlations were not so large as to preclude including all three terms as covariates in a GLMM due to colinearity problems: running each model with one covariate at a time produced comparable effect sizes for focal terms.

More importantly, we needed to take into account that the mean development time differed significantly between the diets because of catch up growth by males on the restricted diet (GLMM with diet as the single fixed factor, and random factors as below: $P < 0.001$). Including development time as a raw covariate could obscure a main effect of diet (i.e. it is a covariate measured post-treatment *sensu* A Gelman and J Hill [75], p.188 that might causally mediate any diet effect because it varies due to the diet itself). We therefore standardised developmental time *within* each diet treatment (both treatments: mean = 0, S.D =1). We also standardised male standard length *within* each diet for ease of interpretation of the results. However, male size did not differ between the diet treatments ($P = 0.451$; see Results) so the use of unstandardised male size yields almost identical results. In contrast, adult age was not standardised *within* diet treatments because it varied depending on when we were able to test males. Instead we standardised adult age *across* the study to aid in interpretation (i.e. the intercept is the value for an average aged male; [76]). Although mean adult age at testing differed significantly between the two diets, there was a large overlap in values (Additional file 1).

Centring the covariates within each diet affects their interpretation. The effect of development time (or its interaction with diet) should be interpreted relative to that of other males on the same diet. The main effects of diet are interpretable as those for a male of average size and development time for its treatment type, but of average age for males across the entire study (see [76]).

In all the GLMMs we specified a Gaussian error distribution and checked the distribution of model residuals to ensure this was appropriate. The use of Poisson error (for count data) and binomial error (for proportions) provided a worse fit to the data than the use of Gaussian error on raw or transformed dependent variables. Each model was fitted using the *lme4* package in *R 3.0.2* software with block, maternal identity, and sire identity as random factors (see [64]). All model terms were tested for significance using the Anova function in the *car* package specifying Type III Wald chi-square tests. Model simplification (i.e. removing non-significant interactions and main terms) did not change our results. Marginal R^2 refers to the variance explained by fixed factors in a model, estimated on the link scale [77]. We present the marginal R^2 (ΔR^2) to show the decline when each fixed effect was dropped from the full model.

We also calculated the effect size (Hedges' *g*) as the standardized difference between males on the control and restricted diets for the measured traits following Rosenberg et al. [78].

Figures are presented using raw data but with model estimates for regression lines, unless otherwise stated. Summary statistics are presented as mean ± SE.

Results

The correlations between the four ejaculate traits are provided in Table 1. Diet treatment means for the five male traits and effect sizes for diet are provided in Table 2. Parameter estimates from the GLMM models are provided in Table 3.

Table 1 Correlations between sperm traits measured

	Number of sperm at day 1	Proportion of sperm replenished	Sperm velocity (VAP, μm/s)
Number of sperm at day 0	0.468 (<0.001)	−0.117 (0.015)	0.055 (0.271)
Number of sperm at day 1		0.664 (<0.001)	0.032 (0.526)
Proportion of sperm replenished			0.059 (0.246)

Estimates are followed by *p*-values in brackets. $N = 452$ males or 393 males (for sperm velocity)

Table 2 Treatment means ± SE (N) for the five traits measured

	Control diet	Restricted diet	Hedges' *g*
Gonopodium length (mm)	6.94 ± 0.05 (223)	7.03 ± 0.04 (226)	0.137
Number of sperm at day 0 ($\times 10^5$)	194.58 ± 6.37 (225)	176.20 ± 6.14 (227)	0.196
Number of sperm at day 1 ($\times 10^5$)	62.36 ± 2.72 (225)	47.92 ± 2.67 (227)	0.356
Proportion of sperm replenished	0.35 ± 0.02 (225)	0.29 ± 0.01 (227)	0.268
Sperm velocity (VAP, μm/s)	83.10 ± 1.11 (207)	81.88 ± 1.12 (186)	0.073

Effect of treatment on male morphology

There was no significant difference between the diet treatments in male body size at maturity (control: 23.52 ± 0.14 mm; restricted diet: 23.35 ± 0.11 mm; $t = 0.92$, $P = 0.36$). Against expectations (see [56]), males on the restricted diet had a significantly *longer* gonopodium than those on the control diet if they were of average or smaller body size, but a shorter gonopodium if they were of above average size (diet × size: $P < 0.001$; Table 3; Fig. 1). Correcting for body size, males that took relatively longer to mature on a given diet did not have a significantly longer gonopodium ($P = 0.110$), irrespective of the diet type (diet × development time: $P = 0.074$).

What influences sperm traits?

Initial sperm reserves (Day 0)

As expected, larger males had significantly greater initial sperm reserves ($P < 0.001$), irrespective of their diet (diet × size interaction: $P = 0.964$). However, diet significantly influenced how sperm reserves changed with age (diet x adult age: $P < 0.001$). Initial sperm reserves of males on the control diet tended to decrease with age ($P = 0.067$), while there was a significant increase with age for males on the restricted diet (Estimate ± SE for males on the restricted diet: 33.777 ± 9.306, $P < 0.001$; Fig. 2).

Although the two slopes intersect, interpretation of the age-dependent change in sperm reserves is best confined to stating that, when younger, males on the restricted diet have lower sperm reserves than those on the control diet. This is because there are relatively few data points for males on the restricted diet when standardised male age exceeds 1 (see Fig. 2). There was also a significant effect of development time on initial sperm reserves ($P = 0.013$), irrespective of diet (diet × development time: $P = 0.132$).

Sperm replenishment rate (Day 1)

Larger males had significantly higher sperm replenishment rates than smaller males ($P < 0.001$), irrespective of their diet (diet × size: $P = 0.868$); but, controlling for body size, males that took longer to develop had a significantly lower sperm replenishment rate ($P < 0.001$) irrespective of their diet (diet × development time: $P = 0.157$). A male of average size and development time for its diet, and average age for the study, that was reared on the control rather than the restricted diet had a significantly higher replenishment rate ($P = 0.005$). There was, however, also a significant difference between the diets in how age related to replenishment rate (diet x adult age: $P = 0.012$): males on the control diet had no significant change in replenishment rate with age ($P = 0.241$; Table 3), while replenishment rate increased significantly with age for males on the restricted diet (Estimate ± SE for males on the restricted diet: 9.245 ± 3.977, $P = 0.020$).

Overall, males on the control diet replenished a significantly greater proportion of their initial sperm reserves within 24 h than those on the restricted diet ($P = 0.014$). The proportion replenished was significantly greater for larger males ($P = 0.022$), and for males with a shorter development time for their diet type ($P < 0.001$), but there was no significant effect of male age ($P = 0.764$). All these relationships held irrespective of diet (interactions with diet: all $P > 0.632$).

Finally, males on the control diet had significantly faster swimming sperm than did males on the restricted diet ($P = 0.006$). Sperm velocity also decreased significantly with adult age ($P < 0.001$; Fig. 3), but was unrelated to development time or body size. All these relationships held irrespective of diet (interactions with diet: all $P > 0.220$).

Discussion

Nutritional constraints early in life can lower an individual's fitness due to changes in their adult performance. Like many species [5, 24], juvenile mosquitofish that experience food restrictions early in life extend their development time to attain a similar body size to individuals on a regular diet (see [55, 56]). But do additional costs arise despite this equivalence in adult body size? Here we tested whether a poor juvenile diet has sexually selected costs for male *Gambusia holbrooki* due to a decline in ejaculate quality and the development of shorter genitalia. We found that early diet had a significant influence on initial sperm reserves, sperm replenishment rate, the proportion of sperm replenished, and on sperm velocity. Shortly after maturation males that had a restricted diet during development had smaller sperm reserves and lower sperm replenishment rates early in

Table 3 Results from mixed models with parameter estimates and chi square (χ^2) tests for food treatment, size, developmental time, and adult age

	Predictor	Estimate	SE	χ^2	*P*	ΔR^2
Gonopodium length [ln (mm)]						
Control diet (N = 223)	Intercept	0.838	0.002	152650	<0.001	
Restricted diet (N = 226)	Diet (restricted)	0.008	0.002	12.670	**<0.001**	0.047
	Size	0.041	0.002	410.140	**<0.001**	0.332
	Developmental time	0.003	0.002	2.553	0.110	0.032
	Diet × Size	−0.020	0.003	43.952	**<0.001**	0.032
	Diet × Developmental time	0.005	0.003	3.193	0.074	0.002
Number of sperm at day 0 (total count $\times 10^5$)						
	Intercept	197.389	8.502	538.991	<0.001	
Control diet (N = 225)	Diet (restricted)	−10.103	8.904	1.2875	0.257	0.036
Restricted diet (N = 227)	Size	25.761	6.978	13.627	**<0.001**	0.040
	Adult age	−19.425	10.606	3.3546	0.067	0.033
	Developmental time	−26.608	10.768	6.1057	**0.013**	0.009
	Diet × Size	0.517	11.465	0.002	0.964	<0.001
	Diet × Adult age	53.202	13.371	15.831	**<0.001**	0.028
	Diet × Developmental time	22.339	14.83	2.269	0.132	0.004
Number of sperm at day 1 (total count $\times 10^5$)						
	Intercept	62.4092	3.1771	385.8565	<0.001	
Control diet (N = 225)	Diet (restricted)	−10.9804	3.8738	8.0347	**0.005**	0.023
Restricted diet (N = 227)	Size	12.9494	3.0323	18.237	**<0.001**	0.057
	Adult age	−5.3597	4.568	1.3767	0.241	0.012
	Developmental time	−21.5316	4.8045	20.0842	**<0.001**	0.045
	Diet × Size	0.8841	5.3062	0.0278	0.868	<0.001
	Diet × Adult age	14.6051	5.8465	6.2404	**0.012**	0.01
	Diet × Developmental time	9.4495	6.6831	1.9992	0.157	0.003
Proportion of sperm replenished						
Control diet (N = 225)	Intercept	219.386	9.188	570.1931	<0.001	
Restricted diet (N = 227)	Diet (restricted)	−30.524	12.415	6.0454	**0.014**	0.013
	Size	22.047	9.611	5.2624	**0.022**	0.02
	Adult age	−4.278	14.272	0.0898	0.764	<0.001
	Developmental time	−55.801	15.094	13.6668	**<0.001**	0.047
	Diet × Size	6.203	16.712	0.1378	0.711	<0.001
	Diet × Adult age	8.97	18.707	0.23	0.632	<0.001

Table 3 Results from mixed models with parameter estimates and chi square (χ^2) tests for food treatment, size, developmental time, and adult age *(Continued)*

	Diet × Developmental time	9.292	21.251	0.1912	0.662	<0.001
Sperm velocity (VAP, μm/s)						
Control diet (N = 207)	Intercept	85.578	1.261	4603.423	<0.001	
Restricted diet (N = 186)	Diet (restricted)	−4.569	1.652	7.650	**0.006**	0.034
	Size	−0.308	1.312	0.055	0.814	<0.001
	Adult age	−8.066	1.932	17.426	**<0.001**	0.062
	Developmental time	−0.293	1.999	0.022	0.883	0.007
	Diet × Size	1.552	2.334	0.442	0.506	<0.001
	Diet × Adult age	2.552	2.529	1.018	0.313	0.003
	Diet × Developmental time	−3.568	2.909	1.505	0.220	0.003

P-values in bold indicate significant values. Covariates were standardised within food treatment. The sample sizes for control and restricted diets are given for each response variable. ΔR^2 shows the change in marginal R^2 when each fixed effect is dropped from the model

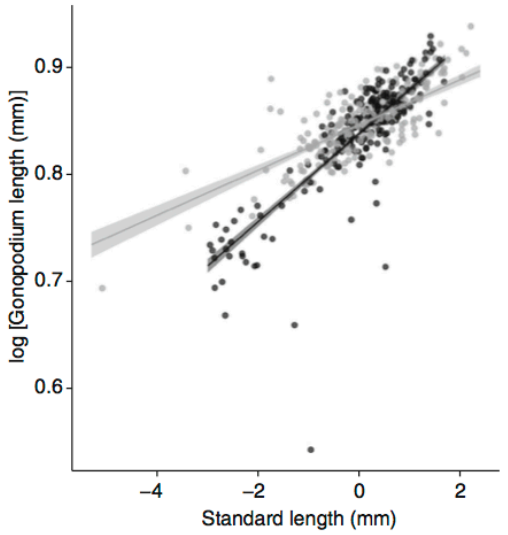

Fig. 1 Effect of diet treatment on the relationship between male body size and gonopodium length. Standard length is standardised within each diet. *Black* symbols and lines represent the control diet, *grey* symbols and lines represent the restricted diet. *Lines* represent simple model predictions. *Grey* shading represents 95% confidence intervals

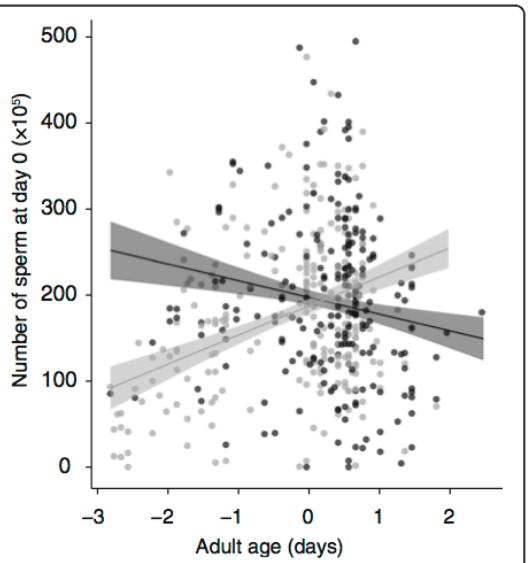

Fig. 2 Effect of diet treatment on the relationship between number of sperm at day 0 and adult age. Number of sperm represent total counts, adult age is standardised across diet. *Black* symbols and *lines* represent the control diet, *grey* symbols and *lines* represent the restricted diet. *Lines* represent simple model predictions. *Grey* shading represents 95% confidence intervals

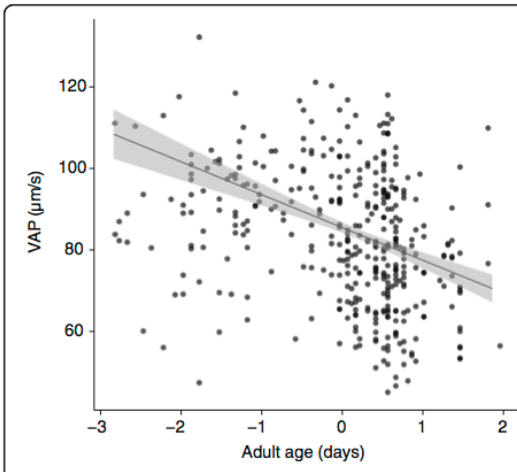

Fig. 3 The relationship between VAP (average path velocity) and adult age. VAP is given in μm/s, adult age is standardised across diet. The *line* represents model predictions. *Grey* shading represents 95% confidence intervals

adulthood than males that did not have a restricted diet. However, these effects were not detectable for older males (see Fig. 2). In contrast, and unexpectedly, males on the control diet had relatively shorter gonopodia than those on the restricted diet, when they were of small or average body size. Our results, combined with those from our previous studies [56, 57], suggest that a poor diet early in life not only has the immediate cost of delayed maturation, but might impose additional costs if lower sperm production, slower swimming sperm, and deviations from the normal gonopodium-body size allometry reduce male fertilisation success under sperm competition.

Sperm production is condition dependent in a variety of species. When the diet of adult males is restricted they tend to have smaller sperm reserves (e.g. [44, 73, 79–81]) and lower sperm replenishment rates (e.g. [54]). There are, however, far fewer studies that explore the effects of a poor juvenile diet on sperm reserves and sperm replenishment rates (but see: [35, 40]). We found that both sperm reserves and replenishment rates were affected by a male's early diet in an age-dependent manner. Thus, in addition to the immediate condition-dependence of sperm production reported in previous studies, we have shown that early diet restriction can have much longer-term effects on sperm production. Whether the relatively small (albeit significant) effects that diet has on sperm production translate into differences in reproductive success remains to be tested. The effect size for the direct effect of diet on sperm production and reserves ranges from $g = 0.20$ to 0.36. To put this in context, by convention, effects of a 0.1

and 0.3 standard deviation change in means are usually described as 'small' and'medium' respectively [82].

Although many factors affect ejaculate competitiveness under sperm competition, sperm number still tends to predict variation in male fertilization success under sperm competition [83–85]. For example, it is a good predictor of fertilization success in another poeciliid fish, the guppy [51]. More generally, a consistent pattern in comparative analyses of diverse taxa is that species with more intense sperm competition have larger testes, and produce more sperm [86–88]. This strongly suggests that sperm production is sexually selected under sperm competition. Our results imply that males that experience a restricted juvenile diet, even if they mate as often as males that had a regular diet, will have lower lifetime reproductive success due to reduced sperm competitiveness. Although sperm reserves increase with time since sexual maturity (here and [89]), males experiencing restricted diets during development may still be disadvantaged because they take longer to reach full sperm production. Additionally, it is worth noting that at our study site adult mosquitofish males do not overwinter [90]. The limited time available to breed (November-March) should favour males that reach full sperm reserves at maturation. However, although we believe that these arguments are compelling, to confirm that the lower sperm production we have reported affects male fitness we still need direct tests of fertilization success whereby males reared on a restricted and normal juvenile diets compete. It would also be interesting to look at how early diet influences the composition of seminal fluids, as this may influence sperm competiveness, for instance by altering sperm longevity ([91], but see [92]) and thus the potential for sperm to be stored over winter.

Our findings for juvenile dietary effects on ejaculatory traits are analogous to those in studies showing that variation in early nutrition due to parental care affects other adult sexual traits that determine male reproductive success [93–95]. For example, in a dung beetle, developing larvae depend on nutrients provided by their parents which affects male body and horn size and thereby their mating success [93]. In some cases the diet or conditions that parents' experience is transmitted to their own offspring (i.e., transgenerational effects: [1, 96, 97]), and can thereby affect their mating success. For example, in birds the amount of carotenoids available to mothers influences what they can deposit into egg yolks, which can then affect their sons' adult ornamental coloration [98]. Our results highlight that, regardless of whether variation in the early nutritional environment is determined by parental care, an individual's resource acquisition ability, or the habitat into which it is born, the effects on offspring fitness are potentially far reaching, and could extend into adulthood. More specifically, we suggest that it could be worthwhile to test for parental effects on sperm production.

Intriguingly, sperm velocity decreases with adult age in *G. holbrooki*. Sperm quality is expected to decline with age due to lower fertilising efficiency and/or the genetic quality of sperm produced by ageing males [99]. This expectation is supported by studies showing that sperm velocity deteriorates with male age (e.g. [100–102]). The lower sperm quality of older males has been shown to reduce fertilization success under sperm competition in some cases (i.e. when competing with sperm from younger males; e.g. [103]) but not others [92, 104]. There are two possible reasons why older males might have lower quality sperm. One is that old males *produce* lower quality sperm (an effect of male age per se; e.g. [100, 105]). The other is that the sperm of older males deteriorates because it has spent more time in storage (an effect of sperm age; e.g. [106, 107]). In our study all sperm velocity measures were obtained from sperm that were at most three days old, so the observed lower sperm velocity is most likely due to an effect of male rather than sperm age. It is intriguing that sperm numbers increased with age (at least for males on the restricted diet), while sperm velocity declined with age for all males. This suggests that sperm number might be a more important determinant of fertilization success than sperm velocity, and is therefore more likely to be maintained given limited resources. This is supported by data from other poeciliids showing that sperm number is more important than sperm velocity under post-copulatory sexual selection (an effect of sperm age; e.g. [106, 107]). Additionally, unlike studies in other poecillids (see [43]), sperm velocity was significantly negatively affected by a restricted diet, albeit that the effect size was small (Hedges' $g = 0.07$). This finding is nonetheless interesting given that we only manipulated diet early in life. Again, however, it remains to be directly shown that the observed dietary difference in sperm velocity affects male fertilization success under sperm competition.

Male fitness depends on the ability to acquire mates and gain paternity when females mate multiply (see [39, 43]). Although the quantity and the quality of sperm tends to strongly influence male fertilization success in most taxa, other traits can be important (e.g. genital morphology in dung beetles; [108]). We measured gonopodium length, which affects female mate choice in some poeciliids and has been implicated as a potentially important trait affecting sperm transfer [109–112]. Unexpectedly, we found that small and medium-sized males on a restricted diet early in life had a longer gonopodium, corrected for body size, than those on a regular diet [60, 113]. The fitness consequences of this change in allometry are unclear. A female preference for males with a relatively longer gonopodium has been shown in

G. holbrooki, but only for large bodied males (see [56] for a different finding). In addition, [109] failed to detect a female preference when using lines of males artificially selected for a relatively larger or shorter gonopodium. Insemination success seems to depend on both male body size and gonopodium length. Males with a relatively longer gonopodium are likely to be more successful, but only when they are large bodied [114]. Paternity studies of males free to compete for females have, however, produced contradictory results. Two studies [61, 62] found that males with a relatively longer gonopodium gained a greater share of paternity, while another study [63] found no difference in the reproductive success of males from lines selected for a relatively longer or shorter gonopodium. Consequently, the effects on male reproductive success of the observed diet-dependent change in relative gonopodium length remain unclear.

Conclusions

In sum, some ejaculate traits in *G. holbrooki* depend on an interaction between a male's juvenile diet and his adult age. In a previous study we also showed that early life diet influences male attractiveness in *G. holbrooki* [57]. Together these studies suggest that early diet could have fitness consequences that only become apparent in adulthood. Our findings are similar to those in other species where males on different diets superficially look the same, but differ in social dominance [57], telomere length or plasma antioxidant levels (e.g. [13]). As with these studies it is assumed that the traits affected by diet influence male fitness. However, the actual effects of a poor early diet on adult male reproductive performance remain to be directly tested. Ideally, future studies should directly measure the relative reproductive success of males that undergo a poor start in life in a competitive mating context (but see [62]).

Additional file

Additional file 1: Shows the overlap in values of adult age between the two diets. (DOCX 49 kb)

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Availability of data and material
Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.k86m5.

Authors' contributions
RV-T participated in the design of the study, performed the sperm section of the laboratory work, performed the statistical analysis, and drafted the manuscript. MDJ participated in the design of the study, assisted in statistical analysis, and helped to draft the manuscript. MLH participated in the design of the study, assisted in statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
This work was conducted under the ANU animal ethics protocol, granted by animal use permit: ANU AEEC animal ethics protocol A2011/64. Collection permits were not required for this study as *G. holbrooki* are a pest species in Australia.

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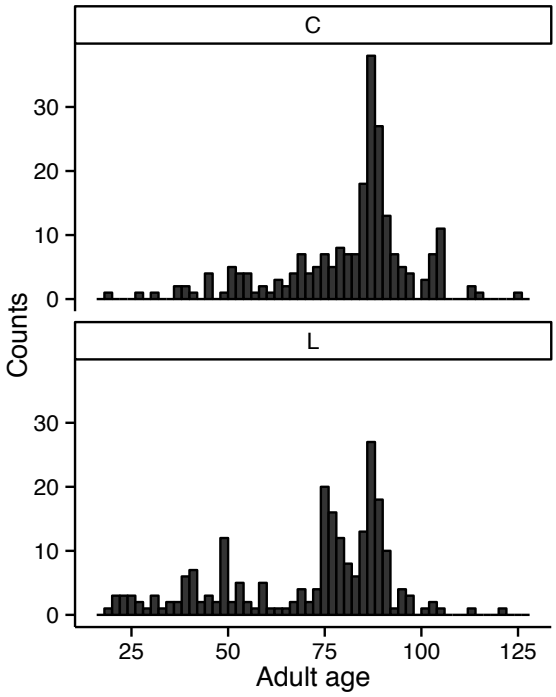
Additional file 1

Histogram of adult age (days since maturation) for control diet (C) and low food diet (L) males.



Chapter 5

Experimental evidence for sexual selection against inbred males

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Experimental evidence for sexual selection against inbred males

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Summary

1. The detrimental effects of matings between relatives are well known. However, few studies determine the extent to which inbreeding depression in males is due to natural or sexual selection. Importantly, measuring fitness or key fitness components, rather than phenotypic traits allows more accurate estimation of inbreeding depression.

2. We investigate how differences in inbreeding and juvenile diet (i.e. early stressful environment) influence a key component of male fitness, namely their reproductive success.

3. We experimentally created inbred and outbred male mosquitofish (*Gambusia holbrooki*) by mating full-sibs (f = 0·25). We show that this led to a 23% reduction in genome-wide heterozygosity based on SNPs. Males were raised on different diets early in life to create high-stress and low-stress rearing environments. We then allowed adult males to compete freely for females to test if inbreeding, early diet and their interaction affect a male's share of paternity.

4. Early diet had no effect on paternity, but outbred males sired almost twice as many offspring as inbred males (n = 628 offspring from 122 potential sires). Using artificial insemination methods we determined that this was unlikely to be due to early embryo mortality of eggs fertilised by inbred males: there was no evidence that male inbreeding status affects the realised fecundity of females (n = 288).

5. Given there was no difference in male mortality in our competitive mating experiment, the lower reproductive success of inbred males can most parsimoniously be attributed to inbreeding negatively affecting sexually selected traits that affect male mating success and/or sperm competitiveness. We discuss which sexually selected traits might be involved.

Key-words: heterozygosity, inbreeding depression, mosquitofish, paternity, reproductive success

Introduction

Environments that are spatially fragmented result in small, isolated populations in which relatives are more likely to mate with each other (Lande 1988; Keller & Waller 2002; Becker *et al.* 2016). Mating between relatives often decreases genome-wide heterozygosity in the resultant offspring, which can reduce the mean phenotypic value of traits putatively associated with fitness, so-called 'inbreeding depression' (Falconer & Mackay 1996; Lynch & Walsh 1998). Inbred individuals are assumed to be less fit due to greater expression of deleterious, recessive alleles (dominance hypothesis) and/or due to homozygosity at loci where heterozygosity confers an advantage (overdominance) (Charlesworth & Charlesworth 1987, 1999). Traits that are closely related to fitness are predicted to be more likely to show inbreeding depression (DeRose & Roff 1999), because strong directional selection promotes fixation of advantageous alleles, and rapidly eliminates deleterious dominant alleles (Lynch & Walsh 1998; DeRose & Roff 1999). By measuring traits that are only weakly related to fitness researchers underestimate the true effects of inbreeding on fitness. More studies are needed that directly quantify the effects of inbreeding on fitness or, given the logistic challenges of measuring net fitness, studies that focus on key fitness components (Hedrick & Kalinowski 2000; Reed & Frankham 2003; Huisman *et al.* 2016).

To date, relatively few experimental studies have looked at the effects of inbreeding on fitness estimates in

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non-domesticated animals. Of these studies, most focus on female reproductive output, or non-sex-specific life-history traits (e.g. Pilakouta & Smiseth 2016), and only a handful have specifically looked at male fitness. For example Zajitschek *et al.* (2009) showed that highly inbred males sire fewer offspring than outbred males; Michalczyk *et al.* (2010) reported that inbreeding depression reduces sperm competitiveness, which can affect male's fertilisation; Konior, Keller & Radwan (2005) estimated the proportion of offspring sired by outbred and inbred males and found that it was lower for outbred males; and Bickley *et al.* (2013) showed a tendency for inbred males to sire fewer offspring when in direct competition with outbred males.

Mating success and fertilisation success under sperm competition are major determinants of male fitness in most species (Andersson 1994; Birkhead & Pizzari 2002; Shuster & Wade 2003). Sexually selected traits that confer a mating or fertilisation advantage are often under strong directional selection and, in addition, they tend to be condition-dependent (Møller 1993; Rowe & Houle 1996; van Oosterhout *et al.* 2003). Condition-dependence has been described as a form of 'genic capture' because condition reflects how well the individual accumulates resources (Rowe & Houle 1996; Tomkins *et al.* 2004). The ability to acquire condition is likely to depend on many traits (e.g. foraging ability, food absorption efficiency) that could be negatively affected by inbreeding. In addition, male-male competition may magnify the effects of inbreeding depression on male reproductive success due to inbred males being weaker competitors or having a poorer ability to obtain territories (Meagher, Penn & Potts 2000; Joron & Brakefield 2003; Yun & Agrawal 2014). It is therefore plausible that, due to sexual selection, male mating success will show greater inbreeding depression than is seen for naturally selected traits that 'capture' less genic variation. These data cannot, however, be obtained from studies that measure male lifetime reproductive output that confound lifespan (i.e. viability selection) with reproductive success per breeding event (i.e. sexual selection).

There is high variation in the reported magnitude of inbreeding depression in the available experimental studies of wild animals that try to measure fitness (e.g. Meagher, Penn & Potts 2000; Harano 2011; Bickley *et al.* 2013; Thonhauser, Raveh & Penn 2014). One possible source of variation is whether or not test individuals experience a stressful environment (Armbruster & Reed 2005; Fox & Reed 2011). Inbreeding might result in individuals less able to buffer their development against environmental stress (Miller 1994). Dietary and temperature stress, for example can increase levels of inbreeding depression (e.g. Dahlgaard & Loeschcke 1997; Kristensen *et al.* 2008; Auld & Henkel 2014; Freitak *et al.* 2014) as can stress arising from intraspecific competition (Meagher, Penn & Potts 2000; Joron & Brakefield 2003; Yun & Agrawal 2014). More generally, rearing animals in a benign laboratory environment (or plants in greenhouses) is often invoked to explain the absence of inbreeding depression in laboratory studies (Duarte *et al.* 2003; Enders & Nunney 2012). Another potential source of variation in estimates of inbreeding depression might be that the evolutionary history of study populations affects the baseline level of heterozygosity. For instance as mean heterozygosity in a population decreases the difference in heterozygosity between offspring of closely related individuals and those from random matings decreases (Pekkala *et al.* 2014). This makes it harder to detect inbreeding depression (see also Miller & Coltman 2014). To date, experimental studies that investigate how these different potential sources of variation influence the effects of inbreeding on fitness-enhancing traits remain scant (but see Dahlgaard & Loeschcke 1997; Reed & Frankham 2003; Pekkala *et al.* 2014).

Here we conduct an experiment to investigate how differences in inbreeding level and juvenile diet (manipulated to create a stressful environment) influence a key component of male fitness, namely reproductive success when competing for mates and fertilisation opportunities in the mosquitofish, *Gambusia holbrooki*. *Gambusia holbrooki* is a poeciliid fish endemic to North America, but now introduced world-wide. Mosquitofish are non-migratory, and are often resident in relatively small bodies of water, such as ponds and streams (Pyke 2005). This makes it likely that inbreeding occurs naturally if a few fish become isolated in a small area. There is sufficient genetic variation in our study population for inbreeding to lead to a detectable, and predicted, decline in heterozygosity (see Results). Mosquitofish have internal fertilisation and males transfer sperm to females via a modified anal fin called the gonopodium (Pyke 2005). Males do not court, but perform coercive 'sneak' copulations in which they approach a female from behind and thrust their gonopodium towards her gonopore (Bisazza & Marin 1995; Langerhans 2011). Male size is highly variable and small males have greater manoeuvrability that seems to increase their propensity to sneak copulations (Pilastro, Giacomello & Bisazza 1997). Large males are, however, socially dominant and might transfer more sperm per encounter because they have larger sperm reserves (O'Dea, Jennions & Head 2014). Female size varies considerably and is strongly correlated with fecundity (Bisazza, Marconato & Marin 1989; Callander, Backwell & Jennions 2012). Females give birth to live young. Finally, standing variation in heterozygosity is positively correlated with male reproductive success when males compete for mates in experimental ponds (Head *et al.* 2016).

We experimentally generated inbred and outbred males that were initially reared on different diets as juveniles (Vega-Trejo, Head & Jennions 2016). We then allowed adult males to compete freely for access to females and quantified their share of paternity. The ability to gain paternity under sperm and mating competition is a key male fitness component in species with high levels of female polyandry, such as *G. holbrooki* (Pilastro,

Giacomello & Bisazza 1997; Bisazza, Vaccari & Pilastro 2001). Importantly, our experimental design allows us to isolate sexual selection (as opposed to other forms of natural selection) as the mechanism driving any inbreeding depression because we eliminated variation in male mortality. In a second experiment we tested, and confirmed, that being inbred did not affect a male's non-competitive fertilisation ability and/or elevate embryo mortality. We established this by artificially inseminating females with either an inbred or an outbred male's sperm and noting their realised fecundity (i.e. offspring at birth). In addition to the experimental manipulation of inbreeding status using a controlled pedigree we directly estimated each male's genome wide heterozygosity (based on >3000 SNPS) to estimate whether the direct use of an actual estimate of heterozygosity provides a more powerful means to detect inbreeding depression than the binary division of males into inbred and outbred. Our design also allowed us to test the prediction that inbreeding depression for reproductive success would be greater for males reared in a stressful juvenile environment.

Materials and methods

ORIGIN AND MAINTENANCE OF FISH

We used mosquitofish descended from wild caught fish collected in Canberra, Australia. The design that we used to create inbred and outbred males that were then reared on different diets, is fully described in Vega-Trejo, Head & Jennions (2016). In brief, in each experimental block we mated individuals from two full sibling families (e.g. A and B in block 1, C and D in block 2,etc). Brothers and sisters from full sibling families were paired to create inbred offspring (AA, BB; $f = 0{\cdot}25$) and outbred offspring with reciprocal male-female crosses (AB, BA) to generate four cross-types. We set up 29 blocks (= maximum of 116 different family pairings types). The 452 male offspring from 192 broods (some experimental blocks had more than one pairing of a given type) were then reared individually in 1 L tanks that were distributed randomly throughout a temperature-controlled room (14 : 10 h photoperiod at 28 °C). Males underwent a diet manipulation for 21 days from day 7 to 28 post birth that lead to almost zero growth (Vega-Trejo, Head & Jennions 2016). Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice daily (i.e. standard laboratory feeding regime), whereas fish on the restricted diet were fed 3 mg of *Artemia* nauplii once every other day (i.e. <25% of the control diet). Broods were split evenly between the control and restricted diet.

EXPERIMENTAL DESIGN – COMPETITIVE MATING SCENARIO

To determine whether inbreeding, diet or their interaction predict paternity we set up mating trials in which four unrelated males, one per treatment, could compete and mate freely with a stock virgin female in a 60 L tank ($n = 31$). Males were randomly assigned to each replicate and were not matched for size (size range: 18·51–26·96 mm). We have previously shown that inbred and outbred males do not differ in size at maturity (Vega-Trejo, Head & Jennions 2016). After a week we removed the female and gave the males a week to recover. The process was then repeated with two more females. The four males in each replicate were kept together for all three trials. The 93 test females were each placed in individual 1 L tanks, and we checked twice daily for 6 weeks whether she had given birth. Offspring were collected immediately and preserved (see below). Adults were killed, preserved in absolute ethanol and stored at −20 °C.

MALE MORPHOLOGY

All males were measured before we placed them in tanks with females. Males exhibit minimal growth after maturation (Cabral & Marques 1999; Pyke 2005; Kahn, Livingston & Jennions 2012), so we did not remeasure them between trials. Males were anaesthetised by submersion in ice-cold water for a few seconds to reduce movement, placed on polystyrene with a microscopic ruler (0·1 mm gradation), and photographed. We measured male standard length (SL = snout tip to base of caudal fin) and gonopodium length (intromittent organ modified from the anal fin) using Image J software (Abramoff, Magelhaes & Ram 2004). The test males were 28–37 weeks post-maturity and were marked with a small coloured dot for visual identification using fluorescent elastomer (Northwest Marine Technology, Shaw Island, WA, USA) injected subcutaneously behind the caudal fin. They had at least 4 days to recover before being placed in 60 L tanks to start mating trials. We calculated relative gonopodium size as the residuals from a linear regression of gonopodium size (log) on SL (log) (Booksmythe *et al.* 2016).

PATERNITY ANALYSIS

To determine male reproductive success and heterozygosity for the fish in our experiment we took tissue samples from each male ($n = 122$), females that bred ($n = 79$ of 93), and up to 10 randomly chosen fry per female ($n = 628$ offspring). In total, 39 of 79 females produced 10 or fewer fry; and we sampled 72% of the total number of fry born (628 of 878).

Two of the 124 males (both outbred) were missing at the end of the trial (i.e. escaped or died) and therefore no tissue was available. DNA was extracted from the tail muscle/caudal fin of adults, and from the whole body, excluding the head, of fry. We used Qiagen DNeasy Blood & Tissue extraction kits following the manufacturer's instructions. After extraction, DNA samples were SNP genotyped. Full methods for the paternity analysis are in the Appendix S1, Supporting Information (see also Booksmythe *et al.* 2016).

HETEROZYGOSITY

We estimated heterozygosity (H) as the number of SNP loci that were scored as heterozygous divided by the total number of successfully classified loci (L) for each male who was a potential sire in the competitive mating experiments (F_{het}). This is essentially a measure of genome wide heterozygosity. F_{het} is identical to $1 - F_{hom}$ in Bérénos *et al.* (2016); and to H/L in Szulkin, Bierne & David (2010, table 2), albeit that there are minor differences in L among individual males; $L = 3360 \pm 2{\cdot}68$ (mean ± SE) loci per male were successfully classified. We found that a brother-sister mating led to a significant decline in offspring F_{het} ($F_{1,120} = 215{\cdot}1$, $P < 0{\cdot}001$) because the proportion of classified

loci per male that were heterozygous was 0·239 ± 0·025 (mean ± SD; range: 0·185–0·288) in inbred males (n = 62) and 0·311 ± 0·028 (mean ± SD; range: 0·263–0·378) in outbred males (n = 60). The mean heterozygosity of inbred fish was therefore 23·2% less than that of outbred fish, close to the expected 25% decline in F_{het}. We also calculated the mean heterozygosity of the 79 females that bred and of the 628 offspring that were genotyped. For the females, F_{het} was 0·314 ± 0·003; and for the offspring, F_{het} was 0·318 ± 0·001. These values do not differ significantly from that for outbred males (one-way ANOVA: $F_{2,763}$ = 2·576, P = 0·077, n = 60 males, 79 females, 628 offspring). There is therefore no detectable sex difference in heterozygosity, and no decline in heterozygosity in the mating trial between outbred individuals in the parental and offspring generation.

EXPERIMENTAL DESIGN — NON-COMPETITIVE MATING SCENARIO

To test whether inbred males have lower non-competitive fertilisation success (i.e. whether eggs were fertilised or not) and/or sired offspring with lower embryo survival we artificially inseminated females with a known quantity of sperm from a single male who was either inbred or outbred (n = 72 inbred, 72 outbred males; split evenly between high and low food diets) and looked at how many offspring the females gave birth to. If observed, we attribute any difference between the two types of males in the number of offspring born to some eggs not being fertilised and/or embryo mortality. Each male was used to inseminate two females from our lab stock population (n total = 288 females). To inseminate females we first anaesthetised the male in iced water, and stripped his sperm (Matthews, Evans & Magurran 1997). To strip sperm males were placed on their side on a glass slide under a dissecting microscope. The gonopodium was swung forward and 100 μL of saline solution (0·9% NaCl) was placed on the slide at the gonopodium tip. Gentle pressure was then applied to the abdomen at the base of the gonopodium so that the ejaculate was released into the saline solution. We used a micropipette to transfer 10 intact sperm bundles (in 3 μL saline solution) directly into the reproductive tract of each of two anaesthetised females. The use of intact sperm bundles results in better fertilisation success than using bundles that have been broken up (Zajitschek *et al.* 2009). After insemination females were housed individually in 1 L tanks, which contained a mesh divider and plastic plants. Females were fed and checked for newborn fry twice daily until they gave birth or until 6 weeks had elapsed. We recorded the number of fry born blind to the inbreeding status of the male.

STATISTICAL ANALYSIS

We used generalised linear mixed-effect models (GLMM) with Poisson error to test for fixed effects of inbreeding, diet, body size, relative gonopodium length and the interaction between inbreeding and diet on how many offspring each male sired. There is no significant effect of inbreeding on relative gonopodium length (GLMM: χ^2 = 0·529; P = 0·467; n = 124). Consequently, including relative gonopodium length in the model does not mask any effects of inbreeding that might act via an effect on gonopodium length (i.e. it is not a covariate measured post-treatment *sensu* Gelman & Hill 2007, p. 188). We used the *glmer* function in the *lme4* package in *R 3.0.2* software (R Development Core Team, 2012). As already noted, Heterozygosity (F_{het}) differed greatly between inbred and outbred males. Our main test for whether heterozygosity affects male success under mating competition is therefore simply the effect of inbreeding status. However, to test whether heterozygosity, after controlling for that associated with inbreeding status, explained additional variation in paternity success, we also standardised heterozygosity. We centred F_{het} so that the mean was 0 for each inbreeding treatment (hereafter F^*_{het}). We then ran the final model including F^*_{het} and its interaction with inbreeding status. An interaction would arise if there is a nonlinear relationship between F_{het} and paternity success. To account for overdispersion we included individual as a random effect (Harrison 2014). Following this correction our data was underdispersed (dispersion parameter = 0·33) and conservative. We included mating trial tank as a random effect to account for potential non-independence. We also included sire and dam identity as random effects in the final model, even though they explained almost no variation in male reproductive success. This can partly be attributed to low statistical power to detect additive genetic variation underlying male reproductive success as, for example of the 60 sires that provided sons we used in the competitive mating trials, the mean number of sons per sire was 2·07 (range 1–6). All fixed model terms were tested for significance using the Anova function in the *car* package specifying Type III Wald chi-square tests. We removed non-significant interactions following Crawley (2005). All tests are two-tailed and alpha is set at 0·05.

To test whether females that were artificially inseminated by inbred males produced fewer broods than those inseminated with sperm from outbred males we used a GLMM with Binomial error. Whether or not a female produced a brood (i.e. 0, 1) was the response variable. Inbreeding status, diet and their interaction were included as fixed factors. We included male identity as a random effect to correct for repeated measurements. We also tested whether male inbreeding status influenced how many fry a female gave birth to. To do so, we used the mean number of fry produced by females (excluding those that did not breed) for each male as the response variable in a GLM with a quasipoisson error structure to account for overdispersion. Male inbreeding status, diet and their interaction were included as fixed factors. We again removed non-significant interactions following Crawley (2005).

To estimate the standardised difference among means we calculated Hedges' g following Rosenberg, Rothstein & Gurevitch (2013). By convention we refer to r = 0·1, 0·3 and 0·5 as small, medium, and large effect sizes respectively (Cohen 1988).

Results

MALE REPRODUCTIVE SUCCESS UNDER A COMPETITIVE MATING SCENARIO

On average, outbred males sired significantly more offspring than inbred males (Table 1, Fig. 1). Outbred males sired 6·37 ± 0·88 offspring, whereas inbred males sired 3·76 ± 0·73 (mean ± SE). This is equivalent to a medium-large effect size of Hedge's g = 0·41. More heterozygous males therefore had significantly greater reproductive success.

Table 1. Results from the mixed model with parameter estimates and chi square (χ^2) tests for heterozygosity, inbreeding, food treatment, size and relative gonopodium size (residuals of the log-log regression of gonopodium length on body size) on the number of offspring males sired. *P*-values in bold indicate significant values (n = 628 offspring genotyped)

	Predictor	Estimate	SE	χ^2	*P*
Number of offspring	Intercept	−17·295	13·888	1·551	0·213
	Relative heterozygosity (F^*_{het})	0·114	0·201	0·319	0·572
	Inbreeding (inbred)	−0·943	0·399	5·596	**0·018**
	Diet (low food)	0·763	0·469	2·643	0·104
	Size [ln(mm)]	12·829	10·004	1·645	0·199
	Relative gonopodium size (residuals)	0·483	0·212	5·179	**0·023**
	Individual identity	3·498			
	Dam identity	0			
	Sire identity	0			
	Mating trial tank	0			

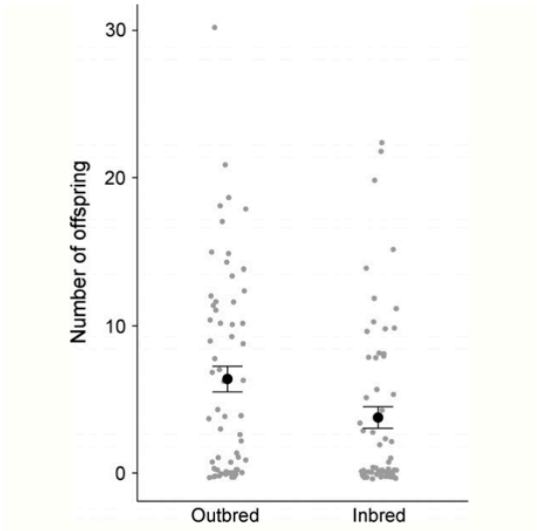

Fig. 1. Mean number of offspring (± SE) sired by outbred and inbred males (n = 122 males genotyped; 60 outbred and 62 inbred). Raw data are represented by dots.

HETEROZYGOSITY CONTROLLING FOR INBREEDING STATUS

We did not find any significant difference in how F^*_{het} affected male reproductive success between inbred and outbred males (F_{het} × inbreeding, χ^2 = 0·873; P = 0·350). There was also no significant effect of F^*_{het} on male reproductive success (Table 1). Together these findings indicate that the residual variation in heterozygosity (i.e. F^*_{het} in outbred males) did not predict variation in male reproductive success. We also tested whether a GLMM using F_{het} was a better predictor of male reproductive success than a GLMM using inbreeding status (the other fixed model terms: diet, body size, relative gonopodium length and an interaction between diet and inbreeding status or F_{het}). The amount of variation explained was identical (R^2 = 0·117), which confirms that in the analysis using inbreeding status and F^*_{het}, the extra information from the use of actual heterozygosity estimates did not allow us to explain significantly more variation that obtained based solely on the difference in heterozygosity generated by the creation of inbred and outbred males.

DIET

We did not find an effect of paternal juvenile diet on the number of offspring sired (Table 1). There was also no significant interaction between inbreeding status and diet (GLMM = χ^2 = 0·297; P = 0·586). The effects of inbreeding were therefore not exacerbated by juvenile diet.

MALE MORPHOLOGY

Males with a relatively longer gonopodium sired significantly more offspring (Table 1). We did not, however, find an effect of male body size on the number of offspring sired (Table 1).

MALE REPRODUCTIVE SUCCESS UNDER A NON-COMPETITIVE MATING SCENARIO

The inbreeding status of males did not affect how many of the females that we artificially inseminated produced offspring, regardless of which diet the males were reared on (Table 2). Forty-eight of 144 females inseminated by an inbred male produced offspring, and 47 of 144 females inseminated by an outbred male produced offspring. Likewise, male inbreeding status did not affect the average number of offspring per brood for females that did breed. Outbred males sired 2·86 ± 0·22 offspring, whereas inbred males sired 3·31 ± 0·25 (mean ± SE; Table 3). There is therefore no evidence that higher early juvenile mortality is biasing our estimate of the share of paternity gained by inbred males downward (i.e. that they fertilised eggs but the offspring died before being counted at birth).

Discussion

Inbreeding is expected to lower fitness due to the negative effects of decreased heterozygosity (Charlesworth & Charlesworth 1987; Lynch & Walsh 1998). Here we used a controlled breeding design combined with a genome wide SNP-based measure of heterozygosity to test whether inbreeding, as well as residual variation in heterozygosity, affects a key component of male fitness, namely reproductive success when males compete for fertilisation opportunities. We found that one generation of inbreeding

Table 2. Results from the mixed model with parameter estimates and chi square (χ^2) tests for inbreeding and food treatment on whether the females that we artificially inseminated produced offspring (n = 288 females)

	Predictor	Estimate	SE	χ^2	P
Number of females that produced broods	Intercept	−0·559	0·217	6·616	0·010
	Inbreeding (inbred)	0·145	0·243	0·356	0·551
	Diet (low food)	0·318	0·244	1·696	0·193
	Male identity	0·034			

Table 3. Results from the generalised linear model with parameter estimates and t tests for inbreeding and diet treatment on the average number of offspring per brood when females were inseminated by a single male who was either inbred or outbred (n = 95 females)

	Predictor	Estimate	SE	t	P
Number of offspring	Intercept	3·452	0·120	28·858	<0·001
	Inbreeding (inbred)	0·140	0·132	1·064	0·290
	Diet (low food)	−0·177	0·132	−1·344	0·182

between full-siblings (f = 0·25), leading to a 23·2% decline in the proportion of SNP loci that were heterozygous, significantly decreased paternity success (6·37 vs. 3·76 offspring per male).

Outbred males sired significantly more offspring than inbred males when they had to compete for mates and fertilisation. This result cannot be attributed to viability selection as only two of 124 males died during the mating trials, and both were outbred. In addition, our artificial insemination study of singly mated females showed that a male's inbreeding status did not affect the likelihood that a female bred, or the number of offspring produced per brood. Inbred males are therefore unlikely to have had a lower estimated share of paternity in our competitive mating trials due to higher embryo mortality, or a naturally selected effect due to lower non-competitive fertilisation ability. Outbred males therefore appear to be favoured when there is sexual selection. Relative gonopodium length, which is not affected by inbreeding, explained some of the remaining variation in reproductive success in a competitive scenario. Males with a longer gonopodium were significantly more successful. We found no evidence that diet or body size affect male reproductive success. Nor did we find any effect of residual variation in heterozygosity once we accounted for the decline in heterozygosity associated with inbreeding in our pedigree design (i.e. the effect of sires' inbreeding status).

HETEROZYGOSITY AND MALE FITNESS

There is indirect evidence from correlational field studies that inbreeding reduces male reproductive success (Chapman & Sheldon 2011; Cain *et al.* 2014; Frère, Chandrasoma & Whiting 2015; Huisman *et al.* 2016). In contrast, studies comparing the reproductive output of experimentally created inbred and outbred males have yielded less consistent results. For example inbreeding depression had no effect on offspring production under a non-competitive scenario in male wild house mice and male flour beetles (Meagher, Penn & Potts 2000; Michalczyk *et al.* 2010), whereas the proportion of offspring sired by inbred males was lower than that of outbred males in bulb mites (*Rhizoglyphus robini*; Konior, Keller & Radwan 2005). In guppies (*Poecilia reticulata*), inbred males sired significantly fewer offspring than outbred males, but only when the inbreeding coefficient was at least f = 0·375 (i.e. two successive generations of full-sib breeding; Zajitschek *et al.* 2009). Inbreeding is, in essence, simply a process that decreases heterozygosity, which is why heterozygosity is used as a proxy for inbreeding (Miller & Coltman 2014; Bérénos *et al.* 2016). Our experiment reveals a significant heterozygosity-fitness correlation (HFC) for male *G. holbrooki*. However, we also show that detecting this HFC could be difficult using standing variation in heterozygosity, as occurs in field studies (Coltman & Slate 2003; Chapman *et al.* 2009; Szulkin, Bierne & David 2010). Specifically, we found no effect of residual heterozygosity (F^*_{het}) on reproductive success for either inbred or outbred males. The variance in (residual) heterozygosity of outbred males in our study should be equivalent to that of males in the field population. [The only caveat is that the variance in heterozygosity in males in the field will be greater if there is inbreeding in the wild. The extent of any difference in heterozygosity will increase with the natural rate of occurrence of inbreeding. We specifically eliminated any such inbreeding in our study by always pairing unrelated fish to create outbred males (Miller & Coltman 2014; Szulkin, Bierne & David 2010)]. It is therefore intriguing that in a new study of field-caught males, albeit with a larger sample (n = 240 putative sires), we detected a significant HFC for male reproductive success when males competed for females in 24 semi-natural pools (M.L. Head, A. Kahn, J.S. Keogh & M.D. Jennions, unpublished data). One interpretation of this difference in the reported effect of heterozygosity is that when males develop under natural field conditions this exacerbates inbreeding depression (see Thrower & Hard 2009). Another possibility is that there is actually considerable variation in the relatedness of mates in the field, which elevates variation in heterozygosity. This source of variation was eliminated in our study due to the controlled breeding design. That is F^*_{het} is heterozygosity after removing effects of parents mating with close relatives.

Studies of inbreeding in the wild generally fail to tease apart natural and sexual selection against inbred males. Reports of lower reproductive success for less heterozygous (i.e. inbred) males could be due to natural selection because of lower rates of survival (e.g. Frommen *et al.*

2008; Mulard *et al.* 2009; Velando, Barros & Moran 2015), which will, all else being equal, reduce their lifetime reproductive success; and/or because inbred males are less attractive to females (including discrimination at the gametic level; Crean & Bonduriansky 2014) or are weaker mating or sperm competitors (Aspi 2000; Meagher, Penn & Potts 2000; Joron & Brakefield 2003; Okada *et al.* 2011). However, sperm traits may not always be affected by inbreeding depression (Mehlis *et al.* 2012; Opatová *et al.* 2016). In our experiment, we can eliminate natural selection through mortality as a major source of variation in male reproductive success (the two male deaths reduce our estimate of inbreeding depression). We can also rule out an effect of male inbreeding status on embryo mortality. When we artificially inseminate virgin females using the sperm of a single male, inbred and outbred males produced the same number of offspring. This finding is similar to studies that have found that the effects of inbreeding depression are not evident under a non-competitive mating scenario (e.g. Meagher, Penn & Potts 2000; Michalczyk *et al.* 2010). Sexual selection is therefore the most likely explanation for the lower reproductive success of inbred males. Indeed, by definition, it is the only explanation (aside from Type 1 error) if sexual selection is broadly defined as variation in reproductive success arising from competition for gametes. It should be noted, however, that competitive interactions in the wild might lead to natural selection on traits that indirectly affect the expression of sexually selected traits (e.g. due to trade-offs in investment) and thereby amplify inbreeding depression on traits under sexual selection.

An obvious question to ask is: what traits account for sexual selection against inbred male *G. holbrooki*? Interestingly, in another study we did not detect inbreeding depression in *G. holbrooki* for sperm traits (velocity and sperm count) or for male attractiveness (based on two-choice association tests), despite much larger sample sizes than in this study (J. Marsh, R. Vega-Trejo, M.D. Jennions & M.L. Head, unpublished data; data and analysis available on request). The lack of inbreeding depression in sperm traits could be attributed to low genetic variation due to founder effects (Ayres, Pettigrove & Hoffmann 2010) because *G. holbrooki* are an introduced feral pest species in Australia. Low genetic variation reduces the magnitude of the difference in heterozygosity between inbred and outbred males. However, the inbreeding depression we report here for actual reproductive success makes this a weak explanation. Ultimately, the results we present here highlight the need to look at how inbreeding affects key fitness components, and not only look at phenotypic traits (such as sperm count) that are only indirect proxies for fitness. Based solely on sperm velocity and sperm count, we would not predict a decline in the fertilisation ability of inbred males. Of course, inbred males might not have less competitive ejaculates. They might simply be less successful at initially inseminating females. In a separate study we used artificial insemination, controlling for sperm number, to test whether inbred males have less competitive ejaculates than outbred males (J. Marsh, R. Vega-Trejo, M.D. Jennions & M.L. Head, unpublished data). There is evidence that the greater the difference in heterozygosity between two competing males the higher the share of paternity gained by the more heterozygous male, suggesting that inbred males will, on average, have less competitive ejaculates.

INBREEDING DEPRESSION IN STRESSFUL AND BENIGN ENVIRONMENTS

Inbreeding depression tends to be higher in a more stressful environment (Armbruster & Reed 2005; Fox & Reed 2011). By definition a more stressful environment is one that reduces fitness relative to a baseline environment (Armbruster & Reed 2005). Our low food diet resulted in almost zero growth over a 3-week period (see Vega-Trejo, Head & Jennions 2016), which strongly suggests that we created a stressful environment. Corroborating this, we have previously shown that this diet significantly reduces male attractiveness measured as female association time (Kahn, Livingston & Jennions 2012). It should, however, be noted that in this study a low food diet did not reduce a male's ability to gain paternity when competing for mates. Studies of other taxa, mainly insects, show that a poor juvenile diet can reduce the ability of males to gain paternity (e.g. Moreau *et al.* 2007). This is mainly attributed to a lower sperm count and reduced sperm competitiveness (Rahman, Kelley & Evans 2013; Muller *et al.* 2015). Elsewhere we have shown that, controlling for age, a poor juvenile diet reduces sperm reserves and sperm replenishment rates in younger male *G. holbrooki* (Vega-Trejo, Jennions & Head 2016). The males in our current experiment were, however, sufficiently old (28–37 weeks post-maturation) that juvenile diets should not have affected sperm production. If sperm number is a major determinant of male reproductive success this would partly explain why there was no main or interactive effect of diet on male success. Again, however, this raises the question of the proximate mechanism causing inbred males to have lower paternity.

Studies of a range of taxa report a weak or no relationship between inbreeding depression and the level of dietary stress (effect size r = -0·13 to 0·02; Fox *et al.* 2011; Reed & Bryant 2001; Reed *et al.* 2003), but most of the focal traits measured in the primary studies are naturally selected. Sexually selected traits that affect male reproductive success are predicted to be more sensitive to inbreeding depression because of their tight links with fitness (Tomkins *et al.* 2004; Drayton *et al.* 2007; Bolund *et al.* 2010; Mallet & Chippindale 2011), and their greater sensitivity to environmental stress because they tend to be condition-dependent (David *et al.* 2000; Ingleby, Hunt & Hosken 2010). It is therefore intriguing that we found significant inbreeding depression for male reproductive success, but no effect of diet. It is possible that we did not

find a dietary effect because the stressful environment was simply not stressful enough or because it was only experienced early in life. More generally, we suggest that studies of many more taxa are needed to establish whether sexually selected traits show the same pattern as naturally selected traits (Armbruster & Reed 2005; Fox & Reed 2011) with respect to whether a more stressful environment elevates inbreeding depression.

MORPHOLOGICAL PREDICTORS OF MALE FITNESS

Males with a relatively long gonopodium for their body size had significantly higher reproductive success in a competitive mating scenario, even taking into account the effects of inbreeding and residual heterozygosity. This corroborates results from another study of *G. holbrooki* in 24 semi-natural pools (M.L. Head, A. Kahn, J.S. Keogh & M.D. Jennions, unpublished data). Several studies of poeciliid fishes have reported a positive correlation between relative gonopodium length and male fitness (Brooks & Caithness 1995; Langerhans, Layman & DeWitt 2005; Devigili *et al.* 2015; Head *et al.* 2015; but see Booksmythe *et al.* 2016). On the other hand male body size, which is often implicated in sexual selection in *G. holbrooki*, had no effect on reproductive success. Previous studies have found mixed results for the effects of male body size (e.g. small male advantage Pilastro, Giacomello & Bisazza 1997; large male advantage Booksmythe, Backwell & Jennions 2013; O'Dea, Jennions & Head 2014) and we suggest that further studies should look into the potential environmental and social factors that might influence this relationship.

Conclusions

We conducted an experiment that showed that inbreeding reduces a key fitness component (share of paternity) of male *G. holbrooki*. Our design removed most sources of natural selection (e.g. offspring and adult survival), and our artificial insemination experiment revealed no effect of male inbreeding on embryo mortality, so the lower reproductive success of inbred males strongly suggests that inbreeding affects sexually selected traits. This is important as sexual selection against inbred males could reduce the genetic load (Enders & Nunney 2012). If inbred males are less likely to mate and/or fertilise eggs, this will reduce the frequency of deleterious recessive alleles and could potentially lower the risk of extinction in small populations (Whitlock 2000; Radwan *et al.* 2004; Sharp & Agrawal 2008; Hollis, Fierst & Houle 2009). This possibility, if generally true in other taxa, could be profitably incorporated into models of population viability, as inbreeding can shape the evolution of key life-history traits (Charpentier, Widdig & Alberts 2007). Of course, we readily acknowledge that our estimate of the effect of inbreeding on males is based on reproductive success in a specific context (four males competing for a female). This is not an unnatural situation given the wide range in adult sex ratios seen in the field (e.g. Cameron 2004; Donald 2007), but the strength of sexual selection might change when there is a less male-biased sex ratio (but see Henshaw, Kahn & Fritzsche 2016).

Our study is a reminder that standing variation in heterozygosity plays an important role in the likelihood of detecting inbreeding depression in correlational studies. This consideration appears to explain variation in reported levels of inbreeding depression, and HFC, in other studies (e.g. Coltman & Slate 2003; Chapman *et al.* 2009; Szulkin, Bierne & David 2010). Residual variation in heterozygosity, hence the use of HFC, was insufficient to detect inbreeding depression in our study: there was no effect of relative heterozygosity (F^*_{het}) on paternity. We only detected inbreeding depression because our breeding design created males with 23% lower than average heterozygosity. Finally, we have to acknowledge the weakness of measuring fitness components in the laboratory. Nonetheless, there is clearly merit in taking an experimental (hence often lab-based) rather than correlational approach to estimate the magnitude of inbreeding depression: experimentally manipulating inbreeding can eliminate the risk of unmeasured confounding factors, that covary with mating partner relatedness, biasing estimates of inbreeding depression (Reid, Arcese & Keller 2008; Becker *et al.* 2016). The ideal study, of course, would experimentally create inbred and outbred males, release them into the wild and then monitor their reproductive success while controlling for natural selection (i.e. mortality). Such studies have, however, to the best of our knowledge not yet been conducted (but see Jimenez *et al.* 1994; Schwartz & Mills 2005).

Authors' contributions

R.V.T., M.L.H. and M.D.J. designed the study. R.V.T carried out the experimental work. J.S.K. analysed the paternity data. R.V.T., M.L.H. and M.D.J. analysed the data and wrote the manuscript. All the authors contributed substantially to revisions, and gave final approval for publication.

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Data accessibility

All data associated with this study have been deposited in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.6d87p (Vega-Trejo *et al.* 2016).

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Supporting Information

Details of electronic Supporting Information are provided below.

Appendix S1. Heterozygosity based on SNPs.

Appendix S1

Heterozygosity based on SNPs

To determine heterozygosity for the fish in our experiment we took tissue samples from a subsample of males (n= 122). DNA was extracted from the tail muscle/caudal fin using Qiagen DNeasy Blood and Tissue Kits following the manufacturer's instructions. After extraction DNA samples were sent to the commercial genotyping service Diversity Arrays. This company has developed a widely used technique called DArTseq™. It represents a combination of DArT complexity reduction methods and next generation sequencing platforms (Kilian et al., 2012; Courtois et al., 2013; Cruz et al., 2013; Raman et al., 2014). It is a new implementation of sequencing complexity reduced representations (Altshuler et al., 2000) on next generation sequencing platforms (Baird et al., 2008; Elshire et al., 2011). It is optimized for each organism by selecting the most appropriate complexity reduction method based on both the size of the representation and the fraction of a genome selected for assays. Four methods of complexity reduction were tested in *Gambusia* (data not presented) and the PstI-HpaII method was selected. DNA samples were processed in digestion/ligation reactions principally following the methods of (Kilian et al., 2012), but replacing a single PstI-compatible adaptor with two different adaptors each corresponding to different Restriction Enzyme (RE) overhangs. The PstI-compatible adapter was designed to include Illumina flowcell attachment sequence, sequencing primer sequence and "staggered", varying length barcode region similar to the sequence reported by (Elshire et al., 2011). The reverse adapter contained a flowcell attachment region and HpaII-compatible overhang sequence. Only "mixed fragments" (PstI-HpaII) were effectively amplified in 30 rounds of PCR using the following reaction conditions: 1. 94 C for 1 min; 2. 30 cycles of 94 C for 20 sec 58 C for 30 sec 72 C for 45 sec; 3. 72 C for 7 min. After PCR equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2500. The sequencing (single read) was run for 77 cycles. (EBPCRI primer: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' and EBHpaIIpcr primer: 5'-

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCG
C TCTTCC GATCTCGG-3').

Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline the fastq files were processed to filter away poor quality sequences applying more stringent selection criteria to the barcode region than the rest of the sequence. In that way the assignments of the sequences to specific samples carried in the "barcode split" step are very reliable. Approximately 2500000 (+/- 7%) sequences per barcode/sample were used in marker calling in routine DArTseq assay but we applied a more cost effective version of the assay using half of the normal tag number (average of 1.3 million per sample). Finally, identical sequences were collapsed into "fastqcall files". These files were used in the secondary pipeline for DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation) calling algorithms (DArTsoft14). For the current sample, this clean-up process resulted in a comprehensive data set of approximately 3455 SNPs with an average call rate of 97.7% and a reproducibility rate of 99.3%. By comparison, the sample of (Booksmythe et al., 2016) resulted in a data set of approximately 4465 SNPs with an average call rate of 93.5% and a reproducibility rate of 98.8%.

Paternity

From these SNPs we calculated a Hamming Distance Matrix for all 122 putative sires and the 628 offspring to determine paternity. Recent studies show that as few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals based on Hamming Distance values (Hu et al., 2015). Each offspring was lined up against its four potential sires, and Hamming Distance values were compared. The sire was the lowest value was considered a match. We could unambiguously assign paternities for all fry (i.e. the distance was markedly lower for one of the four males). We also checked Hamming distances for potential sample mix-ups. We detected none.

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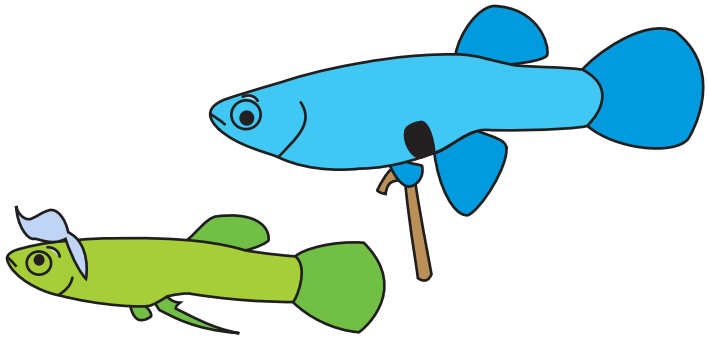
Chapter 6

What happens to offspring when parents are inbred, old or have had a poor start in life?



What happens to offspring when parents are inbred, old or have had a poor start in life?

Regina Vega-Trejo, Loeske E.B. Kruuk, Michael D. Jennions and Megan L. Head

Abstract

Although multiple causes of parental effects on offspring have been documented, we know little about how different aspects interact, or the extent to which they depend on the sex of either the parent or the offspring. We experimentally tested the simultaneous effects of parental age, early diet, inbreeding levels, and their potential interactions on key aspects of offspring development. We found evidence of older mothers producing offspring that were smaller at birth. This negative effect of maternal age persisted throughout life for daughters, but not for sons: the daughters of older mothers matured at a smaller size, albeit sooner. Paternal age did not affect offspring body size, but it had complex effect on sons' relative genital size. When initially raised on a food-restricted diet, older fathers sired sons with relatively smaller genitalia, but when fathers were initially raised on a control diet their sons had relatively larger genitalia. Parental inbreeding had no effect on any of the measured offspring traits. Our results indicate that the manifestation of parental effects can be complex, can vary with both parent and offspring sex, can change over an offspring's life, and is sometimes only evident as an interaction between different parental traits. Understanding this complexity will be important for predicting the role of parental effects in adaptation.

Introduction

The role that parents play in determining the phenotype of their offspring can involve both genetic and non-genetic routes (Kirkpatrick and Lande 1989; Uller 2008; Bonduriansky and Day 2009). The most well-known pathway is that offspring inherit genes that cause them to resemble their parents (i.e. due to additive genetic variation). However, parents can also influence their offspring's phenotype via many other pathways, collectively known as 'parental effects' (Mousseau and Fox 1998; Räsänen and Kruuk 2007; Wolf and Wade 2009). For example, environmental conditions that a parent experiences (e.g. diet or disease), non-additive genetic variation (e.g. parental heterozygosity), parental age, and parental body condition, can all affect an offspring's phenotype (e.g. Annavi et al. 2014; Bouwhuis et al. 2015; Besson et al. 2016). Variation in such parental effects could have substantial implications for offspring fitness, and parental effects on offspring are often of equivalent magnitude to those arising from heritable genetic variation, with potentially large implications for evolutionary and ecological dynamics (Lynch and Walsh 1998; Räsänen and Kruuk 2007; Badyaev and Uller 2009). The full complexity of parental effects is becoming increasingly apparent: identifying the multiple drivers, and determining when, and how, they interact, presents a substantial challenge for evolutionary ecology.

Parental effects can alter offspring morphology, growth, development, and behaviour (Mousseau and Fox 1998; Royle et al. 2012). For instance, parents that are in poor condition, or have been exposed to toxins or other stressors, are likely to produce lower quality offspring (Mousseau and Fox 1998; Uller 2008). But, despite evidence that parental effects are widespread, we still know relatively little about how specific parental phenotypes generate parental effects. Three factors have, however, drawn attention: parental age, parental body condition, and parental inbreeding status. Parental age can have predictable parental effects (e.g. Bouwhuis et al. 2015; Schroeder et al. 2015): there is a trend for older mothers to produce shorter-lived offspring (the "Lansing effect"; Lansing 1947; Hercus and Hoffmann 2000; Priest et al. 2002), and in insects for example, egg hatching rates and larval viability decline with maternal age (Hercus and Hoffmann 2000; Fox et al. 2003; Singh and Omkar 2009). Secondly, parental nutrition affects

offspring phenotype in many species (review: Bonduriansky and Day 2009). This effect is most obvious when there is parental care and food is directly provided by parents to offspring (Smiseth et al. 2012). For example, parental provisioning affects offspring body and horn size in dung beetles (Hunt and Simmons 2000). Additionally, parental nutrition affects offspring phenotype through allocation of resources into eggs (Bernardo 1996; Mousseau and Fox 1998). Finally, inbreeding negatively affects many adult traits ('inbreeding depression'; Keller and Waller 2002), so there is the potential for parental inbreeding status to influence offspring fitness. There is, for example, evidence that more inbred parents produce offspring with lower hatching rates (Mattey et al. 2013; Pooley et al. 2014), reduced body weight (Bérénos et al. 2016), lower juvenile survival (Huisman et al. 2016), weaker immune responses (Reid et al. 2003), and lower reproductive success (Szulkin et al. 2007). Other studies find no evidence that inbred parents produce less fit offspring (Keller et al. 2002). However, despite the increasing evidence for the importance of these three major factors, we lack information about the extent to which they interact to determine paternal effects. For example, are parental effects due to inbreeding heightened in older parents, or those in poor body condition? It is also unclear to what extent the effects of these factors may interact with either the sex of the offspring (i.e. differing between sons and daughters) or that of the parent (i.e. differing maternal or paternal effects).

In species with no post-natal parental care parental effects are confined to the content of eggs and sperm. Here, in general, there is far more evidence for maternal than paternal effects (Curley et al. 2011; Crean et al. 2013). Maternal provisioning of eggs with chemicals and food resources offers a straightforward route whereby mothers can affect early offspring development (e.g. Räsänen and Kruuk 2007; Stynoski et al. 2014). Indeed, variation in the protein and RNA content of eggs is known to directly affect early gene expression in offspring (Fox and Czesak 2000; Johnstone and Lasko 2001; Ducatez et al. 2012). In contrast, it is less clear how paternal effects arise in the absence of male parental care: sperm is mainly considered to be a device to transfer DNA to eggs (Karr et al. 2009; Crean and Bonduriansky 2014). There is, however, increasing evidence that fathers affect the phenotype of their offspring via non-genetic factors even in the absence of direct paternal care (e.g. Bonduriansky and Head 2007; Mashoodh et al. 2012; Crean and Bonduriansky 2014; Fay et al. 2016). For example, a father's diet influences offspring size and age at maturity in springtails (Zizzari et al. 2016). This could be due to the presence

of non-genetic factors in ejaculates, such as lipids and proteins that enter the zygote; or to epigenetic changes in paternal DNA that then affect gene expression in zygotes (Crean and Bonduriansky 2014; Holman and Price 2014). To date, however, studies that directly compare the relative magnitude of maternal and paternal effects are rare, particularly in taxa lacking post-natal parental care.

Here we tested the extent to which parental effects in the eastern mosquitofish (*Gambusia holbrooki*) are shaped by three key characteristics of parents: their age, their own early development, their inbreeding coefficient, and the potential interactions between them. We know that all three factors can shape parental phenotype in other species (e.g. age: Hercus and Hoffmann 2000; diet: Bonduriansky and Head 2007; inbreeding: Mattey et al. 2013), but to what extent do they affect offspring in *G. holbrooki*? Previous work on *G. holbrooki* indicates substantial variation among mothers in maternal effects on these offspring traits (Kruuk et al. 2015) and an effect of inbreeding on reproductive success (Head et al. 2017; Vega-Trejo et al. 2017). Here, we tested the role of three potential causes of this variation, and their potential interactions, in generating both maternal and paternal effects, and quantified their importance for both sons and daughters.

Methods

Study species

The eastern mosquitofish (*Gambusia holbrooki*) is a poeciliid fish endemic to North America, but now found worldwide (Pyke 2005). It was introduced to Australia in 1925, where reported heterozygosity levels are assumed to be lower (Ayres et al. 2010). Native populations of mosquitofish show heterozygosity levels ranging from 0.23 – 0.63 (Vera et al. 2016). Our study population shows heterozygosity levels within the lower end of those seen in the species' native range (mean heterozygosity: 0.27; Head et al. 2017). Gambusia have internal fertilization: females invest in their offspring prior to fertilization by provisioning eggs, but subsequently provide no further contribution (i.e. lecithotrophy; Fernández-Delgado and Rossomanno 1997). Males transfer sperm to females via a

modified anal fin called the gonopodium (Constantz 1989) and mate solely using coercive 'sneak' copulations in which they approach a female from behind and thrust their gonopodium towards her gonopore (Bisazza and Marin 1995; Langerhans 2011). Body size in both males and females has been linked to reproductive success. Smaller males show greater manoeuvrability, which seems to increase their success at sneak copulations (Pilastro et al. 1997; Head et al. 2017), although larger males can dominate their rivals for access to females and might transfer more sperm because they have larger sperm reserves (Bisazza and Marin 1991; O'Dea et al. 2014). Female body size is strongly correlated with fecundity (Bisazza et al. 1989; Callander et al. 2012). Time to reach maturity is highly variable, ranging from 25 to 120 days in our study population (Livingston et al. 2014; Vega-Trejo et al. 2016a). Mosquitofish rarely live longer than 12 to 15 months in the wild, but may live up to 18 months in captivity (Pyke 2008). Finally, heterozygosity has been shown to be positively correlated with male reproductive success, based on both standing variation and experimental manipulation of inbreeding status (Head et al. 2017; Vega-Trejo et al. 2017).

Experimental design

We tested for the impact of parental age, early diet, and inbreeding levels on maternal and paternal effects in two separate experiments. Maternal effects were investigated by mating experimental F_2 females to stock random males, and paternal effects by mating experimental F_2 males to random stock females (see details below). The stock individuals were unrelated to the experimental individuals.

Parental breeding design and fish rearing

Our experimental design consisted of parents who were either inbred or outbred, and were then reared on either a control or restricted diet. Our starting population (F_0) consisted of offspring from 151 gravid wild-caught females collected from three natural ponds around Canberra, Australia (Lake Ginninderra: 35.228°S, 149.063°E, Lake Burley Griffin: 35.289°S, 149.099°E, and Bruce Ponds: 35.241°S, 149.091°E), from October 2009 to April 2010. We inspected fish for maturity to separate males and females. Females

were considered mature when yellow spots were evident in the abdomen, indicating yolked eggs (Stearns 1983). Males were considered to be mature when their gonopodium was translucent, with a spine visible at the tip (Stearns 1983; Zulian et al. 1993). These fish were kept in single-sexed tanks (30-60 fish per 90 L tank) under a 14:10 photoperiod at 28°C, and fed *ad libitum* with *Artemia* nauplii and commercial fish flakes. We then paired males and females randomly from this starting population to create 58 full sib families (F_1; Fig. 1). Fish from these full-sib families were then mated to create an F_2 generation of both inbred (inbreeding coefficient f=0.25) and outbred offspring (Fig. 1). To do this, we used pairs of full-sib families (e.g. family A and family B from block 1, where 'block' represents a pair of families), and created outbred offspring by pairing across families (i.e. female from A and male from B, and male from A and female from B), and inbred offspring by pairing within families (i.e. female and male from A, female and male from B).

We raised a maximum of 10 fish per cross-type individually in 1-L tanks. Individuals from each brood were evenly split between the food treatments: either a control or restricted (low) food diet (see Vega-Trejo et al. 2016a; Fig. 1). Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice a day from birth until the end of the experiment. Fish on the low-food diet were fed the control diet until they were one week old, and were then fed 3mg of *Artemia* nauplii once every other day (i.e. approximately < 25% of the control food intake) for 21 days. We returned them to the control diet after 21 days, so that all fish were on control diet when they were used as parents. An effect of dietary treatment would therefore indicate that parental juvenile development influences the magnitude of parental effects. We then used as parents in our experiment those F_2 fish that survived until maturity and developed normally (e.g. no spinal curvature, which was not related to inbreeding depression, n = 498 of 527 F_2 offspring at birth).

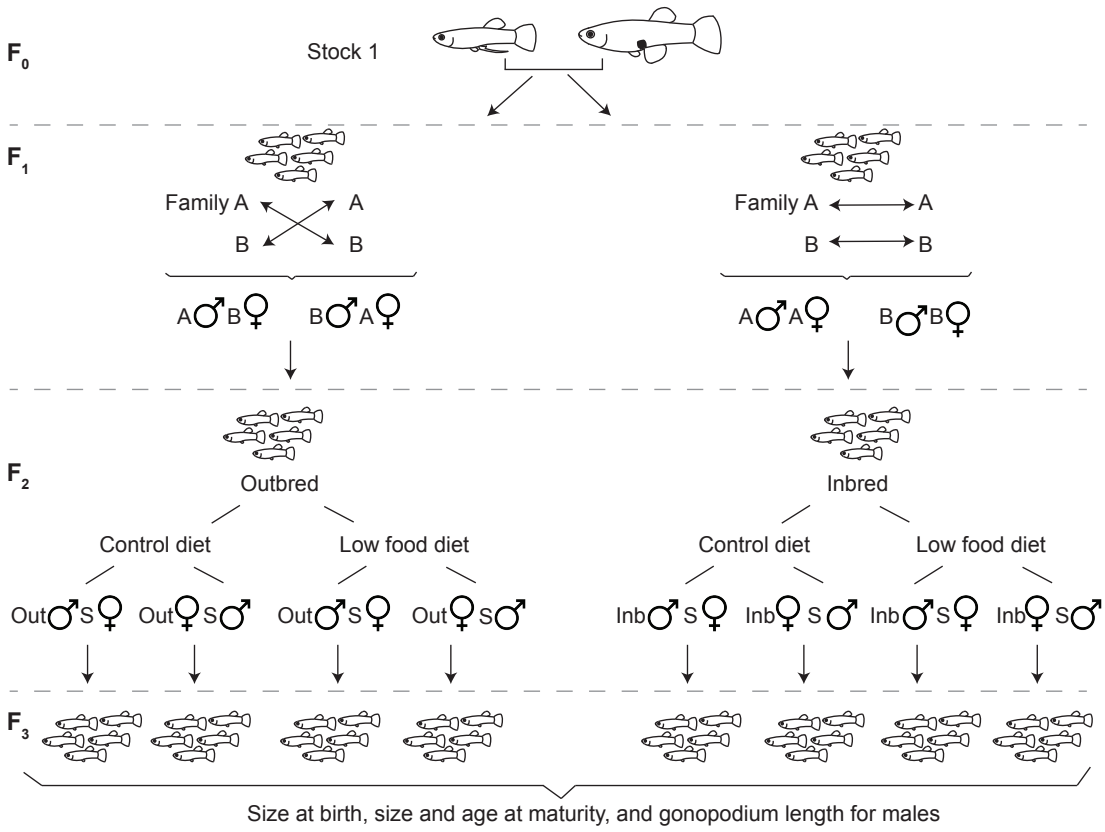


Figure 1. Schematic of the experimental design. Stock 1 = stock fish. S = stock fish unrelated to Stock fish 1. F_0 stock males and females were paired to create F_1 full-sib families (e.g. A and B). We set up 1-4 F_1 females (to maximize the number of offspring) per cross-type to create F_2 outbred (AB, BA; Out) and inbred (AA, BB; Inb) fish. These fish were reared on either a control or a low food diet early in life. F_2 females from each treatment were paired with a stock (outbred) male to create F_3 offspring on which traits were measured. F_2 males from each treatment artificially inseminated stock (outbred) females to create F_3 offspring on which traits were measured. F_2 and F_3 fish were raised individually.

Our design resulted in four parental types as treatments in the F_2 generation (inbred or outbred, reared on a low or control diet), whom we then bred at different ages to generate variation in the third factor of interest, namely parental age. Note that neither inbreeding status nor rearing diet influenced survival to maturity, but both males and females matured later when they were on the low food diet (Vega-Trejo et al. 2016a). Given that age at maturation in *G. holbrooki* is highly variable (see Pyke 2005; Livingston et al. 2014; Vega-Trejo et al. 2016a), we initially considered two measures of parental age

in our analyses: 'chronological age' (days since birth) and 'age since maturity' (days since maturity). The results were qualitatively similar for both, but were consistently clearer for age since maturity. We therefore only present analyses using parental age since maturity in the main text, but analyses and results using chronological age are provided in the online supplement. All data on offspring were collected blind to parental age, diet, and inbreeding status.

Experiment 1: Maternal effects

F_2 females from each of the four treatment groups were mated as virgins to laboratory stock males (n= 94-99 females per treatment). Each female was placed with a single male in a 6.5-L tank for one week to mate. She was then transferred into a separate 1-L tank where we checked for offspring twice daily. Females that did not give birth within six weeks were re-introduced to their original male for another seven days. We recorded female age since maturity (days from maturity until she gave birth), size (standard length, SL in mm), and how many offspring she produced. Means±SD for mother's age since maturity are shown in Table S1. Once females gave birth we individually raised 1-10 (average 4.3 offspring) F_3 offspring per mother in 1-L tanks and recorded their size at birth, and size and age at maturity, and (for sons) gonopodium length (for detailed methods see below). All offspring were fed *ad libitum* with *Artemia* nauplii twice daily.

We obtained data for 945 offspring from 37 outbred/control-diet mothers, 38 inbred/control-diet mothers, 47 outbred/low-diet mothers, and 42 inbred/low-diet mothers. There was no difference in the number of offspring produced by each treatment (linear model: effect of maternal type: $\chi^2_{(3)} = 0.306$).

Experiment 2: Paternal effects

We used artificial insemination in the paternal effects experiment to control for any confounding effects of female preference leading to differential maternal allocation. We took sperm from F_2 males from each of the four treatments (n= 36 males/treatment) and used it to artificially inseminate two laboratory stock females for each male (Fig. 1). To

perform the inseminations, we first anaesthetized the male in ice-cold water. We then placed him on a glass slide with his gonopodium swung forward and put 100µL of saline solution (0.9%NaCl) at the gonopodium tip. We applied gentle pressure to the male's abdomen to expel sperm (Matthews et al. 1997). We then used a micropipette to transfer 10 intact sperm bundles (in 3µL saline solution) directly into the reproductive tract of each of two anaesthetized females. We recorded male age since maturity (days from maturity until he was used to inseminate the females), and how many offspring were produced. Means±SD for male age since maturity are shown in Table S1. We then placed the inseminated females in individual 1-L tanks and allowed them six weeks to give birth, checking for offspring twice daily. We reared a maximum of ten offspring per female in separate 1-L tanks and recorded their size at birth, size and age at maturity, and (for sons) gonopodium length (for detailed methods see below). All offspring were fed *ad libitum* with *Artemia* nauplii twice daily.

We obtained data for 378 offspring sired by 18 outbred/control-diet males, 21 inbred/control-diet males, 27 outbred/low-diet males, and 25 inbred/low-diet males. The number of sires is lower than the maximum possible because only 42% of inseminations were successful (i.e. produced offspring).

Offspring phenotype measurements

To measure offspring size, all offspring were photographed < 18 h after birth. They were placed in a plastic dish (27×27 mm) with 2 mm depth of water to restrict movement and measured from above. To measure their size at maturity, fish were anaesthetized by submersion in ice-cold water for a few seconds to reduce movement then photographed alongside a microscopic ruler (0.1 mm gradation). We used Image J software (Abramoff et al. 2004) to measure standard length (SL = snout tip to base of caudal fin) for both sexes, and gonopodium length (apical tip to base) for males. To determine offspring maturity, we inspected fish three times a week. All inspections for maturity were made blind to treatment. We calculated relative gonopodium size for males as the residuals from a linear regression of (log) gonopodium size on (log) standard length. In total, we measured the following traits on offspring of both sexes: (i) size at birth; (ii) size at maturity; (iii) age at maturity, and (iv), males only: relative gonopodium length.

Statistical analyses

To determine parental effects on offspring traits we ran separate general linear mixed models (GLMM) for each experiment and each trait using the package lme4 (Bates et al. 2015) in R version 3.0.2 (R Development Core Team 2012). We included parental age since maturity (age from maturity to age when the female gave birth or age of when the male was used to inseminate a female), as a covariate, parental diet (control or low), inbreeding status (outbred or inbred), sex of the offspring, and all possible two-way interactions as fixed effects and we specified a Gaussian error structure for all traits given the data distributions. For tractability of interpretation we excluded three-way interactions. Each model was fitted with maternal identity and parental block (i.e. pair of families) as random effects in all models. For the paternal effects analyses we additionally included paternal identity as a random effect because each male could sire up to two broods (33% of the males successfully inseminated two females). Paternal identity was not included in the maternal effects models because each mother was paired with only a single stock male. We standardized all continuous variables (both predictors and dependent) to zero mean and unit variance across the entire data set (i.e. across the maternal and paternal experiment, except for relative gonopodium size—which was standardized separately for the maternal and paternal experiments) prior to analyses to facilitate model convergence and interpretation of the output of models containing interactions. All model terms were tested for significance using the Anova function in the car package specifying Type III Wald chi-square tests, and non-significant interactions were removed. Only final models are presented.

Results

The means (±SE) of the four offspring traits that we measured are given in Table S2, separated by: offspring sex; maternal or parental diet, and maternal or paternal inbreeding status.

Maternal effects

There were no significant differences among the four types of mothers or among mothers of different age in whether or not they produced offspring (generalised linear model with binomial distribution: effect of maternal type: $\chi^2_{(3)} = 0.002$, $P = 0.294$, maternal age: $\chi^2_{(1)} = 3.714$, $P = 0.966$, interaction: $\chi^2_{(3)} = 3.007$, $P = 0.391$).

Older mothers gave birth to significantly smaller offspring, regardless of whether these were sons or daughters ($P = 0.005$; Table 1, Fig. 2). In contrast, the effect that maternal age since maturity had on both offspring size and age at maturation differed between sons and daughters (offspring sex × maternal age interactions: $P = 0.012$ for size at maturation and $P = 0.001$ for age at maturation respectively; Table 1). Daughters of reproductively older mothers were significantly smaller at maturity ($P = 0.014$) and matured significantly earlier ($P < 0.001$), but there were no equivalent effects on sons ($P = 0.760$ and 0.593 for size and age at maturity respectively; Fig. 3a,b).

Neither maternal inbreeding status (all $P > 0.459$), diet (all $P > 0.069$), nor their interaction (all $P > 0.079$) had significant effects on any of the traits (Table 1).

Table 1. Maternal effects on offspring performance: results from Experiment 1. Results from final mixed models with parameter estimates and chi-square2 (χ^2) tests for effects of sex of the offspring, mother's age since maturity, diet, and inbreeding status; non-significant interactions were dropped from the final models. P-values in bold are statistically significant. All analyses were done on standardized response variables. Sample sizes are shown for each response variable. For two-level factors, the parameter shown is the effect of the variable level shown relative to the other.

Response variable	Predictor	Estimate	SE	χ^2	P
Size at birth	Intercept	0.229	0.120	3.651	0.056
(N offspring = 868)	Sex (male)	0.058	0.054	1.156	0.282
(N Mothers = 226)	Mother's age since maturity	-0.179	0.064	7.916	0.005
	Mother's diet (low-food)	-0.034	0.129	0.068	0.794
	Mother's inbreeding status (inbred)	-0.083	0.124	0.454	0.500
Random effects:	Mother's ID variance	0.624			
	Block variance	0.040			
	Residual variance	0.714			
Size at maturity	Intercept	0.719	0.085	71.648	<0.001
(N offspring = 868)	Sex (male)	-0.933	0.055	284.069	<0.001
(N Mothers = 226)	Mother's age since maturity	-0.123	0.050	6.090	0.014
	Mother's diet (low)	-0.081	0.086	0.897	0.344
	Mother's inbreeding status (inbred)	-0.061	0.082	0.549	0.459
	Sex × Mother's age since maturity	0.139	0.056	6.247	0.012
Random effects:	Mother's ID variance	0.164			
	Block variance	0.031			
	Residual variance	0.766			
Age at maturity	Intercept	0.622	0.083	55.569	<0.001
(N offspring = 858)	Sex (male)	-0.984	0.056	305.641	<0.001
(N Mothers = 226)	Mother's age since maturity	-0.167	0.050	11.181	0.001
	Mother's diet (low)	-0.155	0.085	3.317	0.069

	Mother's inbreeding status (inbred)	-0.021	0.082	0.067	0.797
	Sex × Mother's age since maturity	0.195	0.057	11.849	0.001
Random effects:	Mother's ID variance	0.155			
	Block variance	0.026			
	Residual variance	0.777			
Relative gonopodium size	Intercept	0.040	0.095	0.172	0.678
(N offspring = 418)	Mother's age since maturity	-0.086	0.056	2.311	0.129
(N Mothers = 185)	Mother's diet (low)	0.000	0.110	0.000	0.998
	Mother's inbreeding status (inbred)	-0.072	0.105	0.468	0.494
Random effects:	Mother's ID variance	0.055			
	Block variance	0.015			
	Residual variance	0.968			

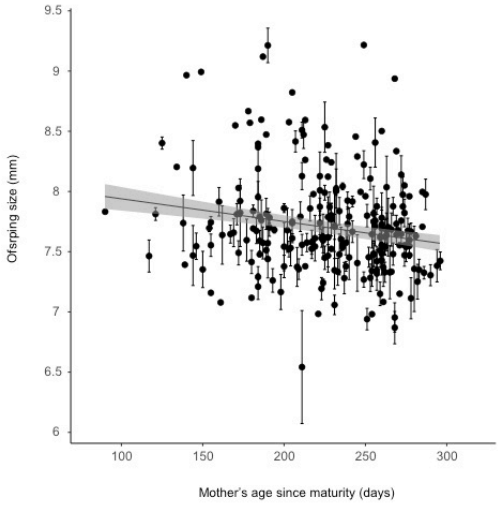


Figure 2. Offspring size at birth. The effect of maternal age since maturity (in days) on the size of offspring at birth. Each data point represents the mean for each family (mothers: N= 226) with SE. The line represents model predictions. Grey shading represents 95% confidence intervals.

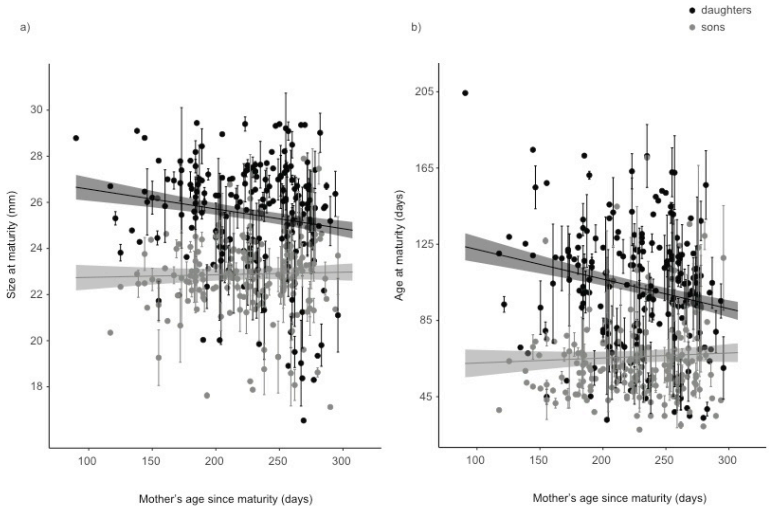


Figure 3. Offspring size and age at maturity. The effect of maternal age on sons' and daughters' a) size and b) age at maturity. Each data point represents the mean for each family (mothers: N = 226) with SE. Black symbols and lines represent daughters, grey symbols and lines represent sons. Lines are based on model predictions. Grey shading represents 95% confidence intervals.

Paternal effects

There was no significant difference among the four types of males or effect of paternal age in whether or not males sired any offspring with either female (generalised linear model with binomial distribution: effect of paternal type: $\chi^2_{(3)} = 0.730$P = 0.866, paternal age: $\chi^2_{(1)} = 0.004$, P = 0.953, interaction: $\chi^2_{(3)} = 1.708$, P = 0.635).

As with mothers, older fathers sired offspring that were smaller at birth, although this effect was marginally non-significant (P = 0.054; Table 2). It was, however, almost identical in magnitude to the effect of maternal age (maternal age -0.179 ± 0.064 SE; paternal age -0.179 ± 0.093 SE). There was no evidence for an effect of father's age on offspring size or age at maturation (both P > 0.275; Table 2). The effect of a father's age since maturity on relative gonopodium length depended on his diet (i.e. interaction between age and diet; P = 0.003; Table 2). Older fathers reared on the low diet sired sons with a significantly shorter gonopodium than younger males (P = 0.029 values for fathers reared on the low diet when the main effect of age is that for fathers reared on the low diet), whereas on the control diet this pattern was opposite and older fathers sired sons with a significantly longer gonopodium than younger males (P = 0.039; Table 2; Fig. 4).

Paternal diet had no effect on offspring size at birth or maturity (both P > 0.241). However, fathers reared on the low diet sired offspring that took longer to mature (P = 0.017; Table 2).

A father's inbreeding status had no effect on offspring size at birth, or their size or age at maturity (all P > 0.322). Inbred fathers sired sons with a relatively shorter gonopodium (P = 0.012; Table 2), but if values more than 2 SD from the mean are excluded (9 of 181 males), the effect is not significant (GLMM: $\chi^2_{(1)} = 2.717$; P = 0.099; n = 172).

Table 2. Paternal effects on offspring performance: results from Experiment 2. Results from final mixed models with parameter estimates and chi-squared (χ^2) tests for effects of sex of the offspring, father's age since maturity, diet, and inbreeding status; non-significant interactions were dropped from final models. P-values in bold are statistically significant. All analyses were done on standardized response variables. Sample sizes are shown for each response variable. For two-level factors, the parameter shown is the effect of the variable level shown relative to the other.

Response variable	Predictor	Estimate	SE	χ^2	P
Size at birth	Intercept	0.290	0.170	2.904	0.088
(N offspring = 344)	Sex (male)	-0.138	0.082	2.878	0.090
(N Fathers = 83)	Father's age since maturity	-0.179	0.093	3.704	0.054
	Father's diet (low)	-0.083	0.184	0.205	0.651
	Father's inbreeding status (inbred)	-0.072	0.156	0.215	0.643
Random effects:	Mother's ID variance	0.383			
	Father's ID variance	0.000			
	Block variance	0.115			
	Residual variance	0.664			
Size at maturity	Intercept	-0.429	0.130	10.906	0.001
(N offspring = 343)	Sex (male)	-0.048	0.087	0.309	0.578
(N Fathers = 84)	Father's age since maturity	-0.033	0.071	0.217	0.642
	Father's diet (low)	0.166	0.141	1.374	0.241
	Father's inbreeding status (inbred)	-0.011	0.118	0.009	0.925
Random effects:	Mother's ID variance	0.108			
	Father's ID variance	0.017			
	Block variance	0.041			
	Residual variance	0.741			
Age at maturity	Intercept	-0.349	0.148	5.542	0.019
(N offspring = 346)	Sex (male)	0.145	0.092	2.469	0.116
(N Fathers = 84)	Father's age since maturity	-0.080	0.074	1.193	0.275

	Father's diet (low)	0.352	0.148	5.674	0.017
	Father's inbreeding status (inbred)	0.119	0.120	0.980	0.322
Random effects:	Mother's ID variance	0.054			
	Father's ID variance	0.024			
	Block variance	0.143			
	Residual variance	0.800			
Relative gonopodium size	Intercept	0.071	0.174	0.168	0.682
(N offspring = 181)	Father's age since maturity	0.344	0.167	4.270	0.039
(N Fathers = 69)	Father's diet (low)	0.014	0.195	0.005	0.942
	Father's inbreeding status (inbred)	-0.424	0.169	6.318	0.012
	Father's diet × Father's age since maturity	-0.627	0.213	8.654	0.003
Random effects:	Mother's ID variance	0.169			
	Father's ID variance	0.000			
	Block variance	0.000			
	Residual variance	0.885			

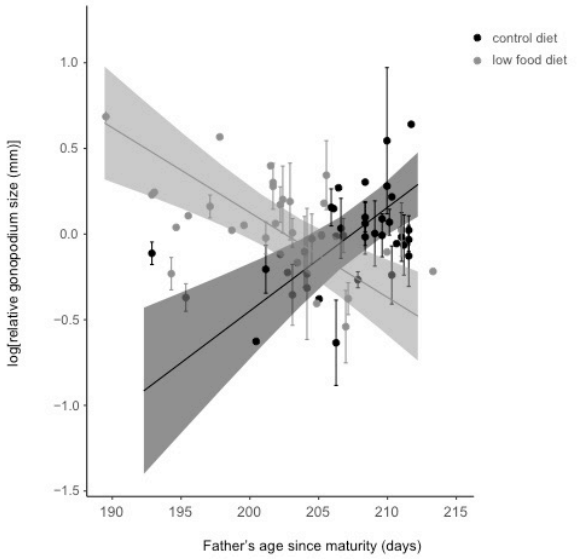


Figure 4. Offspring relative gonopodium size. The effect of paternal age and diet on their offsprings' relative gonopodium size. Each data point represents the mean for each family (fathers: N = 69) with SE. Black symbols and lines represent sons from fathers on the control diet, grey symbols and lines represent sons from fathers on the low food diet. Lines are based on model predictions. Grey shading represents 95% confidence intervals.

Discussion

In this study we explored the action of three factors that generate parental effects in *Gambusia holbrooki*. A mother's age, a father's early diet, and his inbreeding status affected offspring traits such as size at birth, size and age at maturity, and sons' genital length. Parental diet and inbreeding status were both experimentally manipulated so we can assign a direct causal role to each factor. However, the way in which these parental effects occurred was complex. First, the factors, or combinations of factors, causing parental effects differed between mothers and fathers. Second, some parental effects differed for daughters and sons. Third, different factors, or combinations thereof affected how parental effects manifested for each offspring trait we examined.

Comparing maternal and paternal effects: the case of age

Older mothers and older fathers both had offspring that were smaller at birth. Although the paternal effect was marginally non-significant ($P = 0.054$), the estimates for the effect of parental age were remarkably similar to the maternal estimates (see Tables 1 and 2), suggesting that the difference in significance was due to a smaller sample size of fathers. Reports of negative effects of parental age on offspring phenotype are common (e.g. maternal age effects: Hercus and Hoffmann 2000; Benton et al. 2008; paternal age effects: Ducatez et al. 2012; Nystrand and Dowling 2014). Our results are surprising, however, because maternal effects are expected to be stronger than paternal effects when there is no male parental care (Curley et al. 2011; Crean et al. 2013). This is because mothers have greater contact with developing offspring (e.g. gestation) and only eggs contribute substantial material resources to zygotes. Although there is no evidence in *G. holbrooki* that mothers transfer nutrients to offspring after egg fertilization (Pollux et al. 2014), older mothers might provide fewer resources to eggs, thereby affecting offspring birth size. But what about males? Previous studies on poecilids, including mosquitofish, show that sperm quality declines both with paternal age (Vega-Trejo et al. 2016b) and with sperm age (i.e. sperm storage; Gasparini et al. 2010). It is unknown whether this decline is due to changes in ejaculate composition or in the sperm themselves (e.g. epigenetic factors such as DNA methylation), but our results suggest that these changes might be as influential in determining offspring size at birth as those arising from maternal effects (see also Preston et al. 2015). A direct comparison between maternal and paternal effects has to be made with caution given the slightly different structure of the random effects in our models, potential differences in the rearing conditions of the stock fish who were parents in each of our experiments, and differences in how offspring were "created" (i.e. 'natural' matings for maternal effects, and artificial inseminations for paternal effects). Nevertheless, our study adds to recent evidence that paternal effects might be as important as maternal effects in some species (Curley et al. 2011; Crean and Bonduriansky 2014).

Parental effects: sons versus daughters

The effects of maternal age on offspring size and age at maturity were sex-specific. Older mothers had smaller daughters that matured more quickly, but there was no such effect for sons. In mosquitofish, Kruuk et al. (2015) found considerable variation among mothers in maternal effects on the size and time to maturity of both sons and daughters. Intriguingly, however, in that study there was no correlation between the effect a mother had on her sons versus her daughters (e.g. mothers that produced larger daughters did not produce larger sons). Sex-specific maternal effects have also been reported in other species. For example, in seed beetles there were maternal effects on the lifespan of sons, but not of daughters (Fox et al. 2004). Additionally, in red deer there were maternal effects for longevity and breeding success for daughters but not for sons, although these effects might have been driven by shared environmental effects rather than maternal investment (Kruuk et al. 2000). Optimal development differs for males and females due to divergent selection (Uller 2008). Similarly, in the context of sexual conflict, traits that are advantageous in one sex can be detrimental to the other (Kokko and Jennions 2014). Thus, differential investment of parental effects can generate sex-specific effects in their offspring.

Parental effects as offspring age

Although mothers and fathers had similar effects on offspring size at birth, as offspring grew these effects diverged for sons and daughters. In general, the importance of parental effects appears to decline in older offspring (Lindholm et al. 2006; Wilson and Reale 2006). This may be because resources available later in life mask putative parental effects, such as food availability (Monaghan 2008; Auer 2010), or because compensatory growth eliminates initial differences in body size (Metcalfe and Monaghan 2001; Hector and Nakagawa 2012). It is also possible that parental effects actually are stronger earlier when, for instance, maternal investment in egg content still affects offspring (Bernardo 1996). However, we found that, in addition to parental effects being less prevalent in older offspring (Tables 1,2), they tended to become more complex. For example, the negative effect of maternal age on offspring body size and time to maturation only persisted for

daughters, and for fathers, the effect of paternal age on relative gonopodium length was moderated by the father's rearing diet. Although the mechanisms for maternal effects being stronger than paternal effects as offspring age are unknown, it is likely that maternal effects were evident in their offspring later in life because females can adjust their investment to individual offspring when mating to males (Harris and Uller 2009), but that paternal effects arise through direct effects of males themselves. Our results highlight the wider need to account for transgenerational effects when measuring fitness traits (see also Bouwhuis et al. 2015) and how multiple factors can interact in generating parental effects.

Parental effects and inbreeding

The negative effects of inbreeding on individuals' performance are well established (review: Hedrick and Kalinowski 2000; Keller and Waller 2002), so it is tempting to assume that parental inbreeding must have consequences for estimating the total effect of inbreeding of an individual (Huisman et al. 2016). In an earlier experiment we found that inbreeding significantly lowered male reproductive success in *G. holbrooki* (Vega-Trejo et al. 2017) suggesting that it lowers fitness. Even so, there was no detectable inbreeding depression for a range of measured life history (Vega-Trejo et al. 2015; Vega-Trejo et al. 2016a) and reproductive (sperm number, sperm velocity, and gonopodium length; Marsh et al. 2017; Vega-Trejo et al. 2016b) traits in this system. Similarly, there was almost no effect of parental inbreeding status on offspring in the current study. The only exception was that inbred fathers sired sons with a relatively shorter gonopodium, even though they themselves did not have a shorter than average gonopodium (Vega-Trejo et al. 2017). This parental effect could potentially lower sons' fitness as gonopodium length predicts reproductive success in *G. holbrooki* (Head et al. 2017; Vega-Trejo et al. 2017; but see Booksmythe et al. 2016—who showed no fitness cost associated with gonopodium length). In other species of Poeciliids, a shorter gonopodia might be related with male display, or depend on female choice under different predation environments (Bisazza and Pilastro 1997; Langerhans 2011). However, we treat our observed association with sons' gonopodium length with caution given its weak statistical support, and its dependence on nine individuals. It is also worth noting that, to date, most studies showing that parental inbreeding affect offspring phenotypes have all been on species with parental care (e.g. Mattey et al. 2013; Bérénos et al. 2016; Huisman et al. 2016; Pilakouta and Smiseth 2016).

However, some studies have shown parental inbreeding in insects such as *Drosophila* (see Tan et al. 2013; Colines et al. 2015; Nguyen and Moehring 2017).

Conclusion

Parental effects can contribute to the proportion of variance of an individual. Although quantitative genetic studies often consider the proportion of variance due to maternal effects (Falconer and Mackay 1996; Kruuk and Hadfield 2007), both maternal and paternal nongenetic effects can contribute to the characteristics of offspring (Mousseau and Fox 1998; Santure and Spencer 2006). Even so, the underlying causes of variation in parental effects typically remain unknown (Crean and Bonduriansky 2014; van den Heuvel et al. 2016). Here we took an experimental approach to test the extent to which parental inbreeding and diet and variation in parental age alter parental effects. The observed parental effects in *G. holbrooki* depended on both parental and offspring sex, and on interactions between them, age, diet, and inbreeding status. Separating the influence of these factors was facilitated by our simple experimental set up (a two-by-two factorial design) that removed confounding correlations with unmeasured variables. Our study adds evidence for a multifaceted role of parental effects in species lacking parental care and the complexity of factors influencing phenotypic variation. Equally, it raises questions about the proximate mechanisms that generated the observed patterns.

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What happens to offspring when parents are inbred, old or have had a poor start in life?

Supplementary material

Analyses using parental chronological age

In mosquitofish, there is considerable variation in how long it takes individuals to mature. In this data set, the correlation between age since maturity and chronological age was r = 0.365 and r = 0.153 for mothers and fathers, respectively. Because age since maturity and chronological age might influence offspring traits differently (Ligout et al. 2012; Reis et al. 2015), in addition to analysing our data using parental age since maturity, we also analysed it using parental chronological age (for mothers this was the number of days from when a female was born to when she herself gave birth, for fathers this was the number of days from when a male was born to when he was used to artificially inseminate females; see Table S1). The analysis was identical to that described in the main text except that chronological age was substituted for age since maturity.

Results for chronological age – Maternal effects

Using chronological age instead of age since maturity in our models gave very similar results, with one exception. In contrast to our results for maternal age since maturity, there was no interaction between maternal chronological age and offspring sex for size or age at maturity (Table S3).

Results for chronological age – Paternal effects

There were several differences in our results when analysing our data using father's chronological, rather than age since maturity. First, paternal age does not seem to influence offspring size at birth ($P = 0.185$; Table S4). Furthermore, the diet by paternal age interactions for offspring gonopodium length was no longer evident (Table S4). Chronological age, however, shows a significant interaction between paternal age and diet for offspring size at maturity. Lastly, there was no longer an effect of father's inbreeding status on their sons' relative gonopodium length.

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Table S1. Means ± SD (N of offspring/N of parents) from raw data separated by parent's diet treatment for age since maturity and chronological age (both in days). Data shows F_2 parental traits.

	Maternal effects experiment		Paternal effects experiment	
	Mother's age since maturity	Mother's chronological age	Father's age since maturity	Father's chronological age
Outbred control diet	241.25 ± 33.52 (231/54)	312.95 ± 23.97 (231/54)	134.96 ± 32.08 (71/18)	208.16 ± 5.13 (71/18)
Inbred control diet	243.46 ± 34.52 (210/54)	319.37 ± 30.03 (210/54)	144.72 ± 17.46 (123/21)	207.12 ± 3.83 (123/21)
Outbred low diet	223.07 ± 37.09 (256/66)	317.42 ± 24.68 (256/66)	112.56 ± 34.67 (89/26)	203.34 ± 5.62 (89/26)
Inbred low diet	211.56 ± 43.33 (239/58)	315.04 ± 22.92 (239/58)	101.14 ± 24.60 (95/25)	202.54 ± 6.56 (95/25)

Table S2. Means ± SE (N of offspring/N of parents) from raw data separated by offspring sex, parent's inbreeding status, and parent's diet for a) maternal effects experiment and b) paternal effects experiment. Data shows F_3 offspring traits.

a) Maternal effects experiment	Offspring sex		Mother's diet		Mother's inbreeding status	
Offspring trait	Males	Females	Control diet	Low diet	Outbred	Inbred
Length at birth (mm)	7.63 ± 0.02 (422/187)	7.59 ± 0.02 (454/188)	7.61 ± 0.02 (405/108)	7.62 ± 0.02 (463/124)	7.63 ± 0.02 (492/120)	7.59 ± 0.02 (453/112)
Length at maturity (mm)	22.25 ± 0.10 (422/187)	24.95 ± 0.13 (454/188)	23.75 ± 0.14 (405/105)	23.57 ± 0.12 (471/123)	23.79 ± 0.12 (457/117)	23.50 ± 0.14 (419/111)
Age at maturity (days)	64.23 ± 1.32 (421/187)	100.16 ± 1.71 (445/188)	84.35 ± 1.93 (402/105)	81.26 ± 1.61 (464/123)	84.13 ± 1.69 (450/117)	81.13 ± 1.85 (416/111)
Relative gonopodium size (size residuals)	0.00 ± 0.01 (422/187)		-0.007 ± 0.02 (196/85)	0.006 ± 0.02 (226/102)	0.008 ± 0.2 (208/98)	-0.008 ± 0.02 (214/89)
a) Paternal effects experiment	Offspring sex		Mother's diet		Mother's inbreeding status	
Offspring trait	Males	Females	Control diet	Low diet	Outbred	Inbred
Length at birth (mm)	7.55 ± 0.03 (183/68)	7.64 ± 0.03 (161/67)	7.57 ± 0.03 (193/39)	7.58 ± 0.03 (183/50)	7.60 ± 0.03 (160/44)	7.55 ± 0.03 (216/45)
Length at maturity (mm)	22.11 ± 0.15 (181/69)	22.15 ± 0.17 (162/67)	21.96 ± 0.15 (180/38)	22.31 ± 0.18 (163/46)	22.22 ± 0.16 (144/40)	22.60 ± 0.16 (199/44)
Age at maturity (days)	80.82 ± 2.56 (184/69)	75.14 ± 2.33 (162/67)	74.02 ± 2.22 (181/38)	82.70 ± 2.71 (165/46)	77.38 ± 2.53 (144/40)	78.72 ± 2.40 (202/44)
Relative gonopodium size (size residuals)	0.00 ± 0.02 (180/69)		-0.009 ± 0.03 (91/29)	0.009 ± 0.04 (89/40)	0.07 ± 0.04 (78/35)	-0.06 ± 0.03 (102/34)

Table S3. Maternal effects on offspring performance in models using chronological rather than age since maturity (c.f. Table 1). Results from final mixed models with parameter estimates and chi square (χ^2) tests for effects of sex of the offspring, chronological age (age from birth to when the female gave birth), father's diet and inbreeding; non-significant interactions were dropped from final models. P-values in bold are statistically significant. All analyses were done on standardized response variables. P-values in bold indicate significant values. All analyses were done on standardized variables. Sample sizes are shown for each response variable for offspring and for mothers. For two-level factors, the parameter shown is the effect of the variable level shown relative to the other.

Response variable	Predictor	Estimate	SE	X^2	P
Size at birth	Intercept	0.031	0.134	0.054	0.816
(N offspring = 868)	Sex (male)	0.058	0.054	1.158	0.282
(N Mothers = 226)	Mother's chronological age	-0.215	0.058	13.888	<0.001
	Mother's diet (low)	0.304	0.168	3.296	0.069
	Mother's inbreeding status (inbred)	0.185	0.176	1.100	0.294
	Mother's diet (low) × Mother's inbreeding status (inbred)	-0.476	0.241	3.922	0.048
Random effects:	Mother's ID variance	0.589			
	Block variance	0.044			
	Residual variance	0.714			
Size at maturity	Intercept	0.696	0.085	67.476	<0.001
(N offspring = 868)	Sex (male)	-0.931	0.056	280.099	<0.001
(N Mothers = 226)	Mother's chronological age	-0.014	0.040	0.126	0.723
	Mother's diet (low)	-0.046	0.081	0.328	0.567
	Mother's inbreeding status (inbred)	-0.052	0.082	0.401	0.527
Random effects:	Mother's ID variance	0.157			
	Block variance	0.037			
	Residual variance	0.771			
Age at maturity	Intercept	0.592	0.083	50.663	<0.001

(N offspring = 858)	Sex (male)	-0.980	0.057	298.202	<0.001
(N Mothers = 226)	Mother's chronological age	-0.014	0.040	0.129	0.720
	Mother's diet (low)	-0.111	0.081	1.889	0.169
	Mother's inbreeding status (inbred)	-0.011	0.082	0.017	0.897
Random effects:	Mother's ID variance	0.153			
	Block variance	0.029			
	Residual variance	0.784			
Relative gonopodium size	Intercept	0.015	0.095	0.026	0.873
(N offspring = 418)	Mother's chronological age	-0.029	0.052	0.309	0.578
(N Mothers = 185)	Mother's diet (low)	0.047	0.104	0.208	0.648
	Mother's inbreeding status (inbred)	-0.066	0.106	0.386	0.534
	Mother's ID variance	0.047			
	Block variance	0.018			
	Residual variance	0.973			

Table S4. Paternal effects on offspring performance in models using chronological rather than age since maturity (c.f. Table 2). Results from final mixed models with parameter estimates and chi square (χ^2) tests for effects of sex of the offspring, father's chronological age (age from birth to when the female gave birth, diet, and inbreeding status; non-significant interactions were dropped from final models. P-values in bold indicate significant values. All analyses were done on standardized response variables. P-values in bold indicate significant values. All analyses were done on standardized variables. Sample sizes are shown for each response variable. For two-level factors, the parameter shown is the effect of the variable level shown relative to the other.

Response variable	Predictor	Estimate	SE	X^2	P
Size at birth	Intercept	0.137	0.169	0.650	0.420
(N offspring = 344)	Sex (male)	-0.154	0.081	3.563	0.059
(N Fathers = 83)	Father's chronological age	0.118	0.089	1.754	0.185
	Father's diet (low)	0.163	0.166	0.955	0.328
	Father's inbreeding status (inbred)	-0.012	0.159	0.006	0.939
	Mother's ID variance	0.402			
	Father's ID variance	0.000			
	Block variance	0.100			
	Residual variance	0.665			
Size at maturity	Intercept	-0.324	0.132	6.055	0.014
(N offspring = 343)	Sex (male)	-0.067	0.086	0.609	0.435
(N Fathers = 84)	Father's chronological age	**-0.213**	**0.100**	**4.560**	**0.033**
	Father's diet (low)	0.143	0.122	1.362	0.243
	Father's inbreeding status (inbred)	-0.033	0.114	0.083	0.773
	Father's diet × Father's chronological age	**0.359**	**0.128**	**7.917**	**0.005**
	Mother's ID variance	0.102			
	Father's ID variance	0.000			
	Block variance	0.040			

	Residual variance	0.742			
Age at maturity	Intercept	-0.381	0.150	6.441	0.011
(N offspring = 346)	Sex (male)	0.134	0.092	2.126	0.145
(N Fathers = 84)	Father's chronological age	-0.015	0.072	0.045	0.832
	Father's diet (low)	**0.437**	**0.130**	**11.254**	**0.001**
	Father's inbreeding status (inbred)	0.127	0.122	1.076	0.300
	Mother's ID variance	0.052			
	Father's ID variance	0.028			
	Block variance	0.152			
	Residual variance	0.800			
Relative gonopodium size	Intercept	0.246	0.163	2.281	0.131
(N offspring = 181)	Father's chronological age	-0.132	0.094	1.974	0.160
(N Fathers = 69)	Father's diet (low)	-0.115	0.177	0.422	0.516
	Father's inbreeding status (inbred)	-0.326	0.169	3.723	0.054
	Mother's ID variance	0.183			
	Father's ID variance	0.000			
	Block variance	0.000			
	Residual variance	0.896			

Chapter 7

Maternal-by-environment but not genotype-by-environment interactions in a fish without parental care

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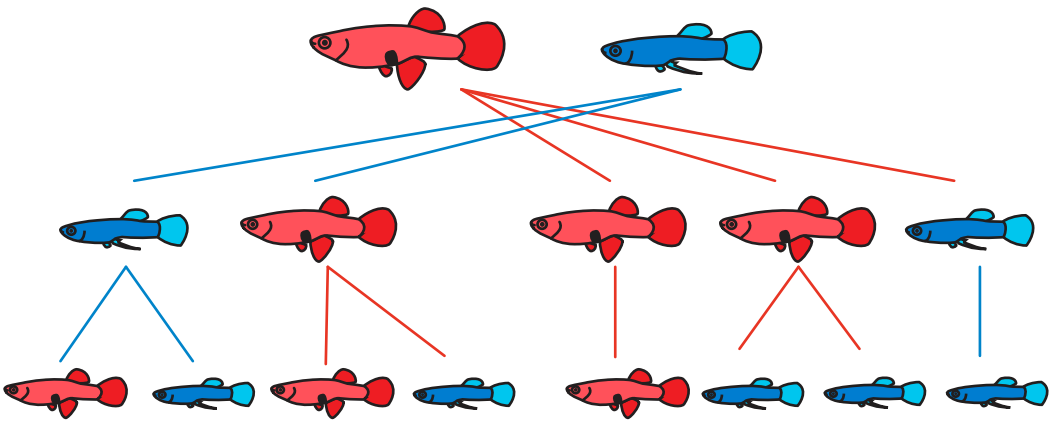

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ARTICLE

Maternal-by-environment but not genotype-by-environment interactions in a fish without parental care

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Abstract
The impact of environmental conditions on the expression of genetic variance and on maternal effects variance remains an important question in evolutionary quantitative genetics. We investigate here the effects of early environment on variation in seven adult life history, morphological, and secondary sexual traits (including sperm characteristics) in a viviparous poeciliid fish, the mosquitofish *Gambusia holbrooki*. Specifically, we manipulated food availability during early development and then assessed additive genetic and maternal effects contributions to the overall phenotypic variance in adults. We found higher heritability for female than male traits, but maternal effects variance for traits in both sexes. An interaction between maternal effects variance and rearing environment affected two adult traits (female age at maturity and male size at maturity), but there was no evidence of trade-offs in maternal effects across environments. Our results illustrate (i) the potential for pre-natal maternal effects to interact with offspring environment during development, potentially affecting traits through to adulthood and (ii) that genotype-by-environment interactions might be overestimated if maternal-by-environment interactions are not accounted for, similar to heritability being overestimated if maternal effects are ignored. We also discuss the potential for dominance genetic variance to contribute to the estimate of maternal effects variance.

Introduction

A central tenet of evolutionary ecology is the expectation that environmental conditions affect evolutionary processes. Evolutionary responses to selection on a trait require the trait to have a genetic basis, so an understanding of the genetic components of phenotypic variation is required to predict evolutionary dynamics (McAdam et al. 2002; Mousseau and Fox 1998; Noble et al. 2014). Quantitative traits are likely to be determined by a large number of genes each with a small effect (Falconer and Mackay 1996; Lynch and Walsh 1998), and the genetic basis of phenotypic variation—or heritability—of a trait can be quantified indirectly from similarities in trait values between relatives (Falconer and Mackay 1996; Lynch and Walsh 1998). However, variation in the environmental conditions individuals experience can play an important role in the process of identifying the genetic components of trait variation. First, similarities between relatives might be due to shared environmental effects, such as maternal effects, which therefore need to be accounted for to generate accurate estimates of heritability (Kruuk and Hadfield 2007; Wolf and Wade 2016). Second, variation in environmental conditions can affect the expression of genetic variance (Rowiński and Rogell 2017; Sgrò and Hoffmann 2004). Third, variation in environmental conditions is also likely to affect the expression of other components of variance, including maternal effects (Mousseau and Fox 1998; Uller et al. 2013).

Changes in the observed genetic variance underlying phenotypic traits in different environmental conditions are known as genotype-by-environment interactions (G × E; Charmantier and Garant 2005; Hoffmann and Merila 1999; Rowiński and Rogell 2017; Wood and Brodie 2015). There

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is abundant evidence from laboratory studies on animals of G × E for a range of traits, based on phenotypic responses to manipulation of environmental conditions such as food availability, temperature, and pathogen levels (e.g. Evans et al. 2015; Ferguson and Read 2002; Vieira et al. 2000). Plant studies also frequently report evidence for G × E (Des Marais et al. 2013): plants of different genotypes or from different populations show marked variation in their phenotypic responses to key environmental variables (e.g. de Leon et al. 2016; Donohue et al. 2000). Understanding whether the performance of genotypes is correlated across environments is critical to determine the extent to which environmental variation might maintain genetic variance (Barton and Turelli 1989; Johnson and Barton 2005): do genotypes that are successful in one environment also do well in another, or are there 'trade-offs' across environments (Kruuk et al. 2008)? Here, we consider how substantial these aspects of G × E might be relative to other determinants of phenotypic variation in key life history and related traits.

An individual's phenotype is shaped by multiple factors in addition to its genotype, one of which is the effect its mother has on it (Pick et al. 2016; Wolf and Wade 2009). 'Maternal effects' occur when a mother's phenotype affects that of her offspring over and above that attributable to the genes it inherits from her (Mousseau and Fox 1998; Räsänen and Kruuk 2007). This might involve pre-natal and/or post-natal effects (Lock et al. 2007; Pick et al. 2016; Wolf et al. 2011). The influence of maternal effects on offspring phenotype is often highly dependent on the mother's own environment. For example, mothers experiencing good environmental conditions may produce larger offspring, breed sooner, or provide more food and greater parental care (Marshall and Uller 2007; Mousseau and Fox 1998; Reznick and Yang 1993). In general, the most obvious mechanisms driving variation in maternal effects point towards environmental factors that alter the mother's phenotype (e.g. mothers in poor condition provide less milk; Trivers 1974). If mothers differ in how they respond to environmental variation, this plasticity can be thought of as "maternal-by-environment interactions" or M × E, akin to genotype-by-environment interactions. Maternal-by-environment (M × E) interactions have been less thoroughly investigated than G × E in the context of variance component analyses (though see for example Chirgwin et al. 2017; Laugen et al. 2005), so in general we have little idea whether 'good' environments generally amplify or depress any differences among mothers, nor of the potential for trade-offs across environments whereby mothers that are superior in one environment are inferior in another (i.e. negative cross-environment maternal effect correlations).

The timing of environmental variation may also drive biologically important effects on trait expression. Maternal effects plasticity is typically investigated in the context of variation in the mother's environment. M × E could, however, equally plausibly be generated by maternal effects differing in interactions with the offspring's environment. In many cases, the two sources of environmental variation impacting on maternal effects are indistinguishable. For example, more stressful environmental conditions reduce variation among mothers in maternal effects for offspring birth weight in wild Soay sheep (Wilson et al. 2005), but it is not possible to determine to what extent this is due to effects of the environment on mothers or on their lambs. We therefore know less about how maternal effects vary 'downstream' due to variation in the *offspring*'s environment. More specifically, are there predictable differences between mothers in how their offspring respond to changes in environmental conditions? In particular, do mothers vary in how much their offspring are able to withstand environmental stress? Addressing this aspect of M × E requires focusing on changes in the offspring's environment once maternal care has ceased.

Analysis of maternal-by-environment interactions may also be important for methodological reasons. Maternal effects typically make relatives (i.e. siblings) look more similar than they would otherwise. They can therefore be difficult to disentangle from additive genetic effects, which are typically estimated from the degree of similarity between relatives. Maternal effects have the potential to inflate estimates of genetic variance unless properly modelled, and there is general acceptance of the need to control for maternal effects when estimating the heritability of a trait (Kruuk and Hadfield 2007; McAdam et al. 2014). However, less attention has been paid to the fact that the same issue applies to tests for G × E interactions: just as the occurrence of maternal effects can inflate estimates of additive genetic variance and heritability, the occurrence of M × E should presumably inflate estimates of G × E. To our knowledge, this possibility remains untested. It implies that, in addition to the fundamental biological question of whether offspring of different mothers are differentially affected by environmental stress due to maternal effects, quantifying M × E may be a critical component of analysis of G × E.

General conclusions about the prevalence of G × E and M × E in a system also require assessment of different traits. Different types of traits typically show different patterns of heritability, of maternal effects and, presumably, of respective interactions with the environment. Traits that are closely associated with fitness often show lower heritability than more weakly-selected traits (Houle 1992; Postma 2014; Roff and Mousseau 1987). For instance, life history traits, such as fecundity and viability, are under strong directional selection and show lower heritability than morphological and physiological traits (Kruuk et al. 2000; Roff

and Mousseau 1987). Sexually selected traits may also show different patterns of variation, alongside differences between the sexes in their genetic architecture (Jia et al. 2000; Parker and Garant 2004). However, comparatively little is known about the relative magnitude of G × E, let alone M × E, for different types of traits.

Here, we experimentally manipulated a critical aspect of the environment experienced by offspring during their early development (food availability), to assess the relative contribution of additive genetic versus maternal effects on phenotypic variance, as well as the extent to which each contribution was influenced by the environmental stress of food restriction. We used a multigenerational breeding design of a laboratory population of mosquitofish (*Gambusia holbrooki*) to test for G × E and M × E interactions in seven adult phenotypic traits: size and age at maturity for both males and females, and three sexually selected male traits, namely, relative genital size, sperm number, and sperm velocity. We considered this range of phenotypic traits in order to investigate the importance of different sources of variance for multiple aspects of adult phenotypes. In many taxa, size and age at maturity are key life history traits often linked to fitness (Roff 1992). Likewise, some sperm traits are strongly positively associated with fitness (Parker and Pizzari 2010), although this is not always the case (e.g. Simmons et al. 2003). We already have clear evidence for maternal effects on growth and development rates that persist until sexual maturity in *G. holbrooki* (Kruuk et al. 2015). Here, we assessed the potential for environmental stress (food restriction) during offspring development to generate both G × E and M × E. *Gambusia holbrooki* is a live-bearing fish lacking post-natal parental care, so all maternal effects must be mediated by events prior to birth. Our experiment therefore constitutes a test for interactions between pre-natal maternal effects (M) and post-natal (i.e. offspring alone) environmental conditions (E). We asked (1) What is the relative importance of additive genetic vs maternal effects in contributing to phenotypic trait variance in each sex? (2) Do these effects interact with the environmental conditions experienced by the offspring? And if so, are there (i) consistent differences in the levels of additive genetic and maternal effects variance between good and poor environments and (ii) trade-offs across environments in either genetic or maternal effects?

Methods

Study species

Gambusia holbrooki, a species of viviparous poeciliid fish, is endemic to North America but now introduced worldwide (Pyke 2005). *G. holbrooki* have internal fertilisation, are sexually dimorphic, and males transfer sperm by a modified anal fin ('gonopodium') that acts as an intromittent organ (Pyke 2005). There is substantial variation in female adult size, which is strongly positively correlated with fecundity (Bisazza et al. 1989; Callander et al. 2012). Male size also varies considerably, despite their growth ceasing at maturation. Small males have greater manoeuvrability, which seems to increase their propensity to sneak copulations (Pilastro et al. 1997), while large males are socially dominant and might transfer more sperm per copulation because they have greater sperm reserves (Bisazza and Marin 1991; O'Dea et al. 2014). Selection on male size in this species is, in summary, unclear. Recent studies in our lab of free-swimming fish have found negative selection (favouring smaller males, Head et al. 2017), no selection (Vega-Trejo et al. 2017), and positive selection (favouring larger males, Booksmythe et al. 2016). Age at maturation in both sexes is highly variable (see Livingston et al. 2014; Pyke 2005; Vega-Trejo et al. 2016a). Greater relative gonopodium size is also linked to increased reproductive success (Head et al. 2017; Vega-Trejo et al. 2017; but see Booksmythe et al. 2016). Finally, sperm velocity declines with age (Vega-Trejo et al. 2016b), and sperm number has been shown to be condition-dependent and positively related to body length (O'Dea et al. 2014).

Experimental design

Our analyses are based on measurements of seven phenotypic traits from laboratory reared *G. holbrooki* in which we varied the level of food an individual received during development. Our multi-generation breeding design also involved the comparison of fish with different levels of inbreeding, as part of a separate investigation into the effects of inbreeding (Vega-Trejo et al. 2016a; Vega-Trejo et al. 2017). The base stock (F_0) population consisted of offspring from 151 gravid wild-caught females collected in Canberra, Australia. F_0 fish were kept in single-sex tanks under a 14:10 photoperiod at 28 °C, and fed *ad libitum* with *Artemia* nauplii and commercial flakes (Fig. 1). Once they were mature, we randomly paired fish from this base stock to create full-sib families (F_1). Fish from these full-sib families were then used to create an F_2 generation consisting of 58 outbred families (with unrelated parents) and 58 inbred families (with full-sib parents; $f = 0.25$; Fig. 1). To do this, we used 29 pairs of full-sib families (e.g. A and B), which we refer to as "blocks". Within each block, one male from family A and one from family B respectively were paired to females (between 1 and 4) from the other family, to create outbred full-sibs/half-sibs (AB and BA); and one male from each family was paired to his full-sib sisters (again, 1–4 females per male) to create inbred full-sibs/half-

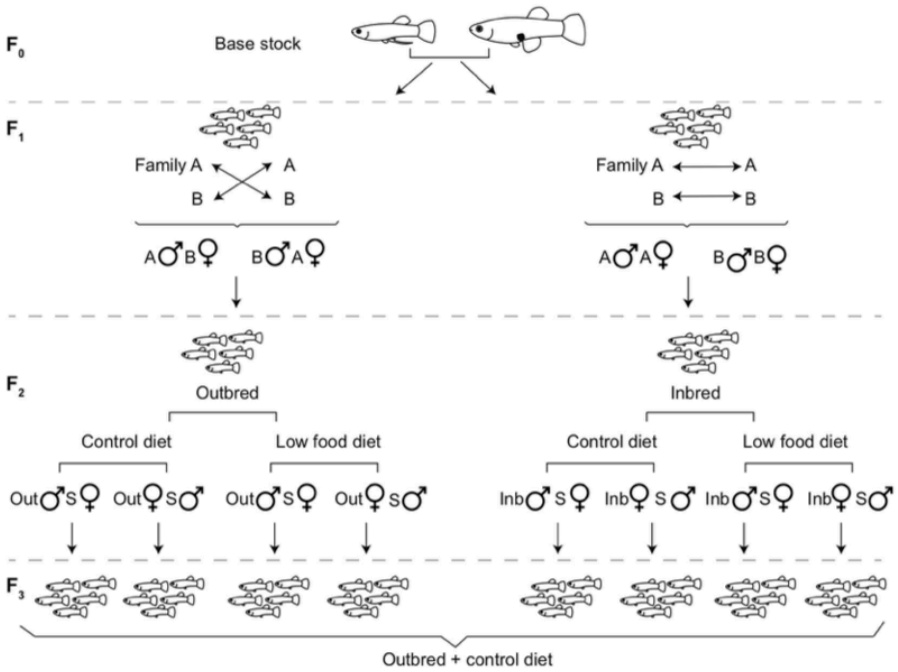

Fig. 1 Schematic of the experimental design. S = stock fish. F_0 stock males and females were paired to create F_1 full-sib families (e.g. A and B). We set up 1–4 females per cross-type to create F_2 outbred (AB, BA—Out) and inbred (AA, BB—Inb) fish. These fish were reared on either a control or a low food diet early in life. F_2 females from each treatment were paired with a stock (outbred) male to create F_3 offspring. F_2 males from each treatment artificially inseminated stock (outbred) females to create F_3 offspring. F_3 offspring were classified as outbred and control diet

sibs (AA and BB). The same number of females contributed to each of the four cross-types within a block to generate a mix of inbred and outbred half-sib and full-sib families. We then reared a maximum of 10 offspring per female (i.e. full-sib family). See Vega-Trejo et al. (2015) and Vega-Trejo et al. (2016a) for a fuller methodological description.

The food manipulation experiment was conducted on the F_2 generation (described below). In the F_2 generation, half of the offspring in each family were raised on a 'control' diet, whereas the other half experienced a 'low food' diet early in life (Fig. 1). Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice a day from birth until the end of the experiment. Fish on the low diet were fed the control diet until they were one week old, and were then fed 3 mg of *Artemia* nauplii once every other day (i.e. <25% of the control food intake) for 21 days, after which they were returned to the control diet. Fish almost totally suppressed growth while on the low food diet (Livingston et al. 2014; Vega-Trejo et al. 2016a). For completeness, we also included in our analyses measurements on the F_3 generation. The F_3 generation was created by pairing each F_2 female with a stock male, and by using sperm from F_2 males to artificially inseminate stock females (Fig. 1). Thus, all F_1 and F_3 fish were outbred and raised on a control diet, whereas F_2 fish were both outbred or inbred, and raised on either a control or restricted diet. Offspring of all generations were transferred to individual tanks at birth to eliminate the potential for post-natal shared environment effects.

Measurements of phenotypic traits

For individuals of generations F_2 and F_3, we measured seven adult traits. Table 1 lists the traits measured, with sample sizes and summary statistics. To determine the timing of sexual maturity, we inspected all tanks three times a week. Females were considered to be mature when yolked eggs were evident in the abdomen (Stearns 1983). Males were considered to be mature when their gonopodium was translucent, with a spine visible at the tip (Stearns 1983; Zulian et al. 1993).

To measure morphological traits, we anaesthetized fish by submersion in ice-cold water for a few seconds to reduce movement. The fish were then photographed alongside a microscopic ruler (0.1 mm gradation). We used Image J software (Abramoff et al. 2004) to measure: *body length* at maturity (snout tip to base of caudal fin, in mm) for both males and females, and male gonopodium length (apical tip to base, in mm). We then calculated *relative gonopodium*

Table 1 Means ± SD (*N*) for the traits measured, and parameter estimates ± SE for the fixed effects are from analyses of standardised values from our basic model $V_A + V_M + V_B + V_R$

Parameter estimates								
Trait	**Trait mean ± SD (N)**	**Intercept**	**Food treatment (low)**	**Inbreeding (inbred)**	**Generation (F_3)**	**Adult age**	**h^2**	**m^2**
Female size at maturity	23.89 ± 2.69 (1035)	0.002 ± .116	−0.368 ± 0.078***	0.058 ± 0.120	0.142 ± 0.106	—	0.309 ± 0.121*	0.254 ± 0.058***
Male size at maturity	22.62 ± 1.93 (1085)	0.325 ± 0.096	−0.192 ± 0.082*	0.076 ± 0.112	−0.507 ± 0.103***	—	0.040 ± 0.092	0.264 ± 0.055***
Female age at maturity	94.25 ± 41.42 (1026)	−0.353 ± 0.110	0.635 ± 0.078***	0.185 ± 0.118	0.398 ± 0.104***	—	0.252 ± 0.113*	0.268 ± 0.054***
Male age at maturity	77.55 ± 34.78 (1060)	0.018 ± 0.094	0.585 ± 0.079***	0.027 ± 0.110	−0.247 ± 0.101**	—	0.000 ± 0.000	0.286 ± 0.039***
Relative gonopodium size	0 ± 0.36 (1053)	−0.481 ± 0.089	0.386 ± 0.089***	0.153 ± 0.102	0.637 ± 0.097***	—	0.039 ± 0.083	0.094 ± 0.047*
Sperm number	186.77 ± 94.53 (442)	0.065 ± 0.101	−0.100 ± 0.093	−0.035 ± 0.100	—	0.137 ± 0.049**	0.000 ± 0.000	0.074 ± 0.052
Sperm velocity	83.01 ± 16.57(390)	0.098 ± 0.094	−0.230 ± 0.096*	0.059 ± 0.103	—	−0.302 ± 0.052***	0.197 ± 0.125	0.016 ± 0.085

In all models, we fitted food treatment (control vs low food diet), inbreeding (inbred vs outbred), and generation (two levels, F_2–F_3). For sperm number and sperm velocity, generation was not included as a fixed effect because we only had data for F_2 males, but we included the age of the male at measurement (measured in days post maturity). Estimates of fixed effect parameters and proportions are followed by their SEs. Heritabilities (h^2) and the proportion of the maternal effects variance (m^2) ± SE are from the basic model with $V_A + V_M + V_B + V_R$. Significance of h^2 and m^2 is given as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (see Methods for details).

size for males as the residuals from a linear regression of (log) gonopodium length on (log) standard length (Horth et al. 2010; Vega-Trejo et al. 2017).

We measured two sperm traits in the F_2 generation: *sperm number* and *sperm velocity*. Details on the extraction of ejaculates and the samples are given in the Supplementary Information. In brief, males were anaesthetized in ice-cold water, placed on a glass slide and their gonopodium was swung forward. We then applied gentle pressure to the abdomen to eject all of the available sperm. We counted the sperm and measured sperm velocity. Afterwards, each male was returned to his individual tank. All inspections for maturity and measurements of traits were made blind to food treatment, inbreeding status, and family identity.

Statistical analyses

We quantified components of variance in the phenotypic traits using an 'animal model', a form of mixed model that uses pedigree information to assess covariance between relatives (Wilson et al. 2010), fitted using ASReml-R (Butler et al. 2009). All models contained random effects of an additive genetic effect (with covariance structure defined by relatedness between individuals, as determined by the breeding design's pedigree, and associated additive genetic variance component V_A), a maternal effect (grouping individuals by mother, with associated maternal effects variance component V_M; Kruuk and Hadfield 2007), a block component (with variance component V_B, defined above), and residual effect (with associated variance component V_R).

The animal model estimates the additive genetic variance underlying a trait's variance based on the similarity between multiple types of relatives; here relatedness, was defined by our four-generation pedigree (F_0–F_3). The estimate of the maternal effects variance V_M was determined by the increased similarity between offspring of the same mother, beyond that due to additive (direct) genetic effects (Kruuk and Hadfield 2007). This value will necessarily encompass both maternal environment effects and maternal genetic effects (i.e. effects of the mother's genotype on her offspring, over and above the direct effects of the genes they inherit from her); we do not attempt to separate them. Offspring were reared separately from birth onward (see above), so there is little potential for post-natal common environment effects to inflate estimates of maternal effects. Offspring of the same mother were, however, always full-siblings, so there is the potential for the estimate of V_M to be inflated by dominance genetic variance (Falconer and Mackay 1996). We make the standard assumption that dominance variance is small compared to additive genetic variance (e.g. Hill et al. 2008; Zhu et al. 2015), and hence

that any impact is also small, but we return to this point in the Discussion.

All traits were standardised to unit variance and zero-centred prior to analysis, and we analysed the data separately for males and females. In all models, we fitted food treatment (control vs low food diet), inbreeding (inbred vs outbred), and generation (two levels, as phenotypic data were only available for F_2 and F_3) as fixed factors. For sperm number and velocity, generation was not fitted because we only measured F_2 males. However, for the sperm traits we included fixed effects of male age (range 19–125 days post maturity) as age influences sperm number and velocity (Vega-Trejo et al. 2016b). The parameter estimates for the fixed effects in a model with only V_A, V_M, V_B, and V_R (see details below) are in Table 1. Parameter estimates for the effects of food treatment are shown in Fig. S1.

Univariate models of interactions with environmental conditions

We first fitted univariate models for each of the seven traits to explore the extent of interactions of both the additive genetic variance and maternal effects variance with environmental conditions (i.e. food treatment). We started with Model 1, a 'null' model with only fixed effects (as described above: food, inbreeding, and generation), block as a random effect, and a residual random effect. We then fitted: Model 2, which included variance due to additive genetic effects (V_A); Model 3, a model containing just the maternal effects variance (V_M); and Model 4, a model that included both components of variance ($V_A + V_M$). We call this the 'basic model'. We report the trait's heritability (V_A / ($V_A + V_M + V_B + V_R$)) and maternal effects proportion (V_M /($V_A + V_M + V_B + V_R$)) from the basic model in Table 1.

We then investigated whether the variance components changed between the two food treatments (i.e. Genotype × Environment or Maternal effect × Environment) by testing for either a $V_A \times$ Food or $V_M \times$ Food interaction. To do so, we first included the interactions between each variance component and the food treatment in models with only one main variance component (i.e. Model 5: $V_A + V_A \times$ Food; and Model 6: $V_M + V_M \times$ Food), and then in models which included both main terms (i.e. Model 7: $V_A + V_M + V_A \times$ Food; and Model 8: $V_A + V_M + V_M \times$ Food). Finally, we ran a model with all main terms and interactions (Model 9: $V_A + V_M + V_A \times$ Food $+ V_M \times$ Food).

Model comparison

Because we wished to compare several models, many of which were not nested, we used a model comparison approach based on the Akaike Information Criterion AIC (Burnham and Anderson 2002; see Saastamoinen et al. 2013 for a similar model comparison of different animal models). We calculated AIC as -2log(L) + 2 K, where log (L) was the model's log likelihood and K the number of parameters estimated. We only considered the number of parameters associated with estimating variance in the different random effects (other than the residual and block variance estimates), given that the fixed effects were the same in all models for a given trait. Thus, K ranged from 0 (null model: model with only fixed effects and one random effect of block) to 4 (final model with V_A, V_M, $V_A \times$ Food and $V_M \times$ Food). Akaike weights w for each model i were calculated as $w_i = \exp(\Delta AIC_i)/\sum\exp(\Delta AIC)$, where ΔAIC is the difference in AIC between model i and the model with the lowest AIC (the top model for that trait). Models ranked within two AIC units of the top model were considered to be 'reasonable candidate' models providing indistinguishable levels of support (Burnham and Anderson 2002). The number of reasonable models ranged from one to four across the different traits.

The informational approach outlined above allowed us to compare differences between models for each trait. We then determined the significance of the interaction parameter estimates in the top model. To do this, we ran likelihood ratio tests (LRT) comparing the top model to one without that parameter. The test statistics and P-values are given in the text of the *Results*. We also tested for the significance of V_A and V_M by LRTs by respectively dropping those terms from the basic model ($V_A + V_M + V_B + V_R$). The P-values from these LRTs are shown in Table 1 adjacent to the heritability (i.e. for V_A) and to the proportion of maternal effects variance (i.e. for V_M).

For each trait, we report the variance components for the reasonable candidate models in the main *Results*, and for all nine models in the Supplementary Information. However, given that there might be some level of support for multiple models, we also provide model-averaged parameter estimates (Burnham and Anderson 2002). For each trait, we model averaged the estimates of the different variance components across our 9 models, where the model-averaged variance component V^* was calculated by weighting V_i, the variance estimate of model i with the weights w_i as calculated above, such that $V^* = \sum (V_i \times w_i)$. We also estimated model-averaged standard error as $SE^* = \sum [w_i \times \sqrt{(SE_i^2 + (V_i - V^*)^2)}]$ (Burnham and Anderson 2002).

Bivariate models across environments

We found evidence for interactions between food treatment and variance components for both female age at maturity and male size at maturity (see *Results*). We therefore fitted bivariate models to estimate the variance due to additive genetic effects and maternal effects within each food

treatment, by splitting each trait by treatment to define two new "sub-traits". These models contained inbreeding level and generation as fixed effects as above, and maternal and additive genetic effects as random effects in order to generate environment-specific estimates of the variance components in each trait. Block was also included as a random effect for female age at maturity, but was not fitted for male size at maturity given a lack of convergence (note: for other traits, fitting block made little difference to the parameter estimates). Each model also contained estimates of the covariance across environments in additive genetic, maternal effect, and (for female age) block effects. We also ran a model with additive genetic effects only to compare our estimates of V_A when maternal effects were ignored.

Results

Almost all traits were affected by the food treatment during development, but there was no effect of inbreeding on trait means (Table 1). However, there were marked differences among traits as to which of the different combinations of variance components produced the best model. Table 2 shows the reasonable candidate models (<2 AIC units from the top model) for each trait with their corresponding variance estimates, and also the model-averaged variance estimates averaged across all models. For the corresponding details of all 9 models for each trait, see Table S1.

We found evidence of additive genetic and maternal effects variance for almost all traits. However, the interaction with food treatment varied substantially between traits. Below we present results for each trait in turn.

Female size at maturity

Females on the low food treatment were slightly smaller at maturity than those on control diets (Table 1; Fig. S1). For the 'basic' model with just $V_A + V_M$, we found substantial heritability and maternal effects variance (Table 1). Comparing all models, both of the 'reasonable candidate' models contained variance due to additive genetic effects and maternal effects, with the top model containing only $V_A + V_M$ (i.e. the 'basic' model as defined above; Table 1). Although $V_M \times$ Food appeared in the second-ranked model, overall there was little indication of support for this interaction (LRT: $X^2_{(1)} = 0.546$, $P = 0.761$; Table 2); this model fits the characteristics of a 'hitch-hiking' model, where an additional term generates a very slight improvement in model fit and so falls within 2 AIC units of the top model (Arnold 2010; Symonds and Moussalli 2011). Similarly, there was no support for a $V_A \times$ Food interaction (LRT: $X^2_{(1)} < 0.001$, $P = 1$; Table S1).

Male size at maturity

Males on the low food treatment were also smaller at maturity (Table 1; Fig. S1). The estimates of heritability and maternal effects proportions from the basic $V_A + V_M$ model showed little heritability but substantial maternal effects variance in male size (Table 1). Model comparison indicated strong support for a $V_M \times$ Food interaction as the term appeared in both of the two reasonable candidate models (Table 2), and the top model contained both V_M and a significant $V_M \times$ Food interaction (LRT: $X^2_{(1)} = 18.205$, $P < 0.001$). We found little support for a $V_A \times$ Food interaction (Table S1). We fitted a bivariate model of environment-specific traits to estimate the variance components for each food treatment. There was significant variance in maternal effects in the control environment but not in the low food environment (Fig. 2a). There was also a negligible covariance (negative) of the maternal effects across environments, most likely because of the lack of variation among mothers in the low food environment (Fig. 2a, Table 3).

Female age at maturity

Females in the low food treatment took on average 21% longer to mature (Table 1; Fig. S1). Similar to female size at maturity, the basic $V_A + V_M$ model indicated substantial heritability and maternal effects for female age at maturity. In the model comparison, the only reasonable candidate model was that containing V_A and V_M and a $V_M \times$ Food interaction (LRT for $V_M \times$ Food: $X^2_{(1)} = 6.449$, $P = 0.039$; Table 2). When we considered female age at maturity in the two environments separately, we found much higher variance in maternal effects in the low food environment than in the control environment, and also a positive covariance of maternal effects across the two environments (Fig. 2b, Table 3).

Male age at maturity

Males in the low food treatment took on average 38% longer to mature than those on the control diet (Table 1, Fig. S1). As with male size at maturity, the basic model with only $V_A + V_M$ showed little heritability but substantial maternal effects variance (Table 1). The reasonable candidate model set contained a top model with V_M only and another with V_M and a $V_M \times$ Food interaction. However, similar to female size, the second model was consistent with a hitch-hiking model because there was little support for the $V_M \times$ Food interaction (LRT: $X^2_{(1)} = 0.987$, $P = 0.611$; Table 2).

Relative gonopodium size

Males in the low food environment had relatively longer gonopodia on average (Table 1, Fig. S1). In the basic model

Table 2 The reasonable candidate models for each trait

Model	log (L)	AIC	Δ AIC	Weight	$V_A \pm SE$	$V_M \pm SE$	$V_A \times Food \pm SE$	$V_M \times Food \pm SE$	$V_B \pm SE$	$V_R \pm SE$
Female size at maturity										
$V_A + V_M$	−417.145	842.291	0	0.456	0.310 ± 0.124	0.255 ± 0.060			0.056 ± 0.041	0.382 ± 0.066
$V_A + V_M + V_M \times$ Food	−416.872	843.745	1.454	0.221	0.321 ± 0.124	0.210 ± 0.078		0.045 ± 0.060	0.056 ± 0.042	0.368 ± 0.067
Model averaged estimates (across all models)					0.290 ± 0.138	0.243 ± 0.075	$5.430 \times 10^{-8} \pm 8.791 \times 10^{-8}$	0.014 ± 0.031	0.056 ± 0.042	0.377 ± 0.073
Male size at maturity										
$V_M + V_M \times$ Food	−466.625	941.249	0	0.574		$3.813 \times 10^{-8} \pm 2.257 \times 10^{-9}$		0.311 ± 0.043	0.019 ± 0.015	0.603 ± 0.036
$V_A + V_M + V_M \times$ Food	−466.239	942.478	1.229	0.310	0.060 ± 0.074	$4.989 \times 10^{-7} \pm 3.839 \times 10^{-8}$		0.284 ± 0.051	0.010 ± 0.018	0.574 ± 0.050
Model averaged estimates (across all models)					0.026 ± 0.050	$3.188 \times 10^{-4} \pm 6.618 \times 10^{-4}$	$2.238 \times 10^{-4} \pm 4.691 \times 10^{-4}$	0.299 ± 0.049	0.015 ± 0.017	0.590 ± 0.044
Female age at maturity										
$V_A + V_M + V_M \times$ Food	−401.233	812.467	0	0.595	0.255 ± 0.112	0.162 ± 0.071		0.137 ± 0.063	0.044 ± 0.037	0.368 ± 0.062
Model averaged estimates (across all models)					0.240 ± 0.119	0.177 ± 0.079	0.003 ± 0.009	0.116 ± 0.074	0.045 ± 0.038	0.373 ± 0.067
Male age at maturity										
V_M	−436.383	878.765	0	0.4154		0.257 ± 0.257			0.023 ± 0.016	0.618 ± 0.035
$V_M + V_M \times$ Food	−435.892	879.783	1.0181	0.2497		0.215 ± 0.061		0.060 ± 0.061	0.023 ± 0.016	0.602 ± 0.037
Model averaged estimates (across all models)					$4.620 \times 10^{-4} \pm 0.010$	0.234 ± 0.142	$1.299 \times 10^{-8} \pm 2.572 \times 10^{-8}$	0.020 ± 0.040	0.023 ± 0.016	0.591 ± 0.044
Relative gonopodium size										
V_M	−501.979	1009.958	0	0.378		0.102 ± 0.035			0.012 ± 0.011	0.839 ± 0.045
$V_A + V_M + V_A \times$ Food	−500.879	1011.757	1.799	0.154	0.019 ± 0.062	0.082 ± 0.042	0.083 ± 0.071		$7.749 \times 10^{-8} \pm 5.965 \times 10^{-9}$	0.766 ± 0.059
$V_A + V_M$	−501.888	1011.776	1.818	0.152	0.037 ± 0.079	0.089 ± 0.045			0.005 ± 0.017	0.820 ± 0.060
$V_M + V_M \times$ Food	−501.931	1011.861	1.903	0.146		0.094 ± 0.050		0.017 ± 0.059	0.012 ± 0.011	0.831 ± 0.050
Model averaged estimates (across all models)					0.013 ± 0.043	0.083 ± 0.046	0.013 ± 0.034	0.003 ± 0.015	0.007 ± 0.012	0.750 ± 0.088
Male sperm number										
V_M	−215.358	436.716	0	0.323		0.073 ± 0.051			0.085 ± 0.042	0.824 ± 0.068
Null	−216.726	437.452	0.736	0.224					0.099 ± 0.042	0.885 ± 0.062

Table 2 (continued)

Model	log (L)	AIC	Δ AIC	Weight	$V_A \pm SE$	$V_M \pm SE$	$V_A \times Food \pm SE$	$V_M \times Food \pm SE$	$V_B \pm SE$	$V_R \pm SE$
Model averaged estimates (across all models)					$1.247 \times 10^{-7} \pm 2.514 \times 10^{-7}$	0.042 ± 0.054	$4.051 \times 10^{9} \pm 1.104 \times 10^{-8}$	$4.542 \times 10^{-8} \pm 7.846 \times 10^{-8}$	0.082 ± 0.044	0.774 ± 0.099
Male sperm velocity										
V_A	−179.474	364.947	0	0.127	0.190 ± 0.085				$7.034 \times 10^{-8} \pm 9.136 \times 10^{-9}$	0.695 ± 0.090
$V_A + V_M$	−179.458	366.915	1.968	0.115	0.175 ± 0.112	0.014 ± 0.075			$7.057 \times 10^{-8} \pm 9.077 \times 10^{-9}$	0.697 ± 0.090
Model averaged estimates (across all models)					0.100 ± 0.115	$0.014 \pm \pm 0.046$	$1.389 \times 10^{-7} \pm 1.928 \times 10^{-7}$	0.009 ± 0.044	0.008 ± 0.017	0.574 ± 0.172

For each model, the log likelihood (log (L)) is presented. Models shown are the models ranked within two AIC units of the top model in descending order. The difference in AIC value with the model with the lowest AIC value is presented (Δ AIC). Weight = Akaike model weight, relative to all 9 models for each trait (see Methods for definition). V_A additive genetic variance, V_M maternal effects variance, $V_B$block variance, V_R residual variance.

with just $V_A + V_M$, the heritability and the strength of maternal effects were both relatively low, though the latter accounted for nearly 10% of the phenotypic variation and was statistically significant (Table 1). The top model contained only V_M. Although models containing both $V_A \times$ Food and $V_M \times$ Food interactions were included in the reasonable candidate model set, neither parameter estimates differed significantly from zero (LRT: $V_A \times$ Food: $X^2_{(1)} = 2.019$, $P = 0.364$; $V_M \times$ Food: $X^2_{(1)} = 0.097$, $P = 0.953$; Table 2).

Sperm traits

Males in the control treatment had sperm with a faster mean velocity, but there was no difference between food treatments in mean sperm number (Table 1; Fig. S1). For sperm number, the top model contained only maternal effects variance V_M, and heritability was negligible (Table 2). However, the estimate of V_M in the top model had a large SE and was not significant (LRT: $X^2_{(1)} = 2.736$, $P = 0.098$) and the null model with only fixed and block effects was also a 'reasonable candidate' model (Table 2). For sperm velocity, the top model contained only V_A, with a corresponding significant heritability of 0.215 (0.092 SE), (LRT: $X^2_{(1)} = 4.715$, $P = 0.029$), but if we considered the 'basic' model with $V_A + V_M$, the estimate of heritability was reduced to 0.197 (0.125 SE; Table 1) and was non-significant (LRT: $X^2_{(1)} = 2.073$, $P = 0.149$).

Discussion

Understanding the causes of phenotypic variation in traits is fundamental to predicting evolutionary responses. Our study of life history and sexual traits in the mosquitofish *Gambusia holbrooki* revealed several patterns. First, we found sex differences in heritabilities: there were significant heritability estimates for female age and size at maturity, but not for the male traits examined. Second, in both sexes there were maternal effects that persisted until sexual maturity for all traits except the sperm traits (i.e. for five of seven traits). Third, there were interactions between maternal effects variance and food treatment for female age at maturity and male size at maturity. Failure to account for maternal by environmental interactions (M × E) led to overestimates of genotype-by-environment interactions (G × E). We discuss each of these points below.

The relative importance of heritable genetic effects in our study differed between males and females: in general, female traits had higher heritability. Because of the lack of genetic variance in males, we did not attempt to estimate cross-sex genetic correlations, but our results indicate a very different underlying genetic architecture shaping female and

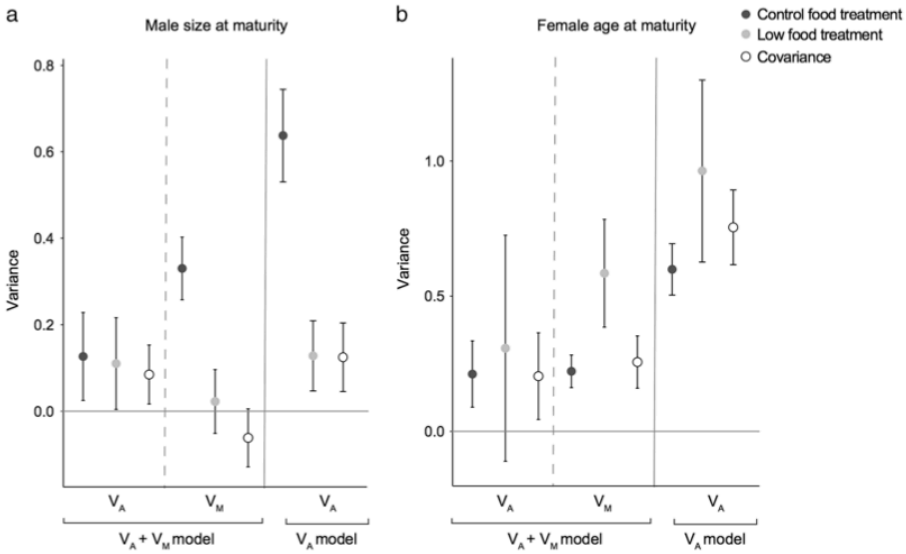

Fig. 2 Effect of food treatment on variance components for **a** male size at maturity; **b** female age at maturity: additive genetic effects (V_A) and maternal effects variance (V_M) ± SE for a bivariate model with $V_A + V_M + V_B + V_R$, and V_A only ($+V_B + V_R$; see Methods for details). Dark symbols represent values for fish in the control food treatment, light symbols represent values for fish in the low food treatment, and white symbols represent the covariance between the traits in the two treatments

Table 3 Variance–covariance matrices from the bivariate models of traits expressed in each environment for female age at maturity and male size at maturity

	Female age at maturity		Male size at maturity	
	Control	Low food	Control	Low food
V_A	0.212 (0.122)	0.800 (0.699)	0.127 (0.102)	0.719 (0.644)
	0.204 (0.160)	0.307 (0.418)	0.085 (0.068)	0.110 (0.106)
V_M	0.222 (0.060)	0.711 (0.256)	0.330 (0.072)	−0.712 (1.378)
	0.256 (0.097)	0.585 (0.199)	−0.062 (0.067)	0.023 (0.074)
V_B	0.049 (0.036)	0.989 (0.776)	–	–
	0.071 (0.055)	0.106 (0.161)	–	–
V_R	0.363 (0.066)	–	0.570 (0.063)	–
	–	0.418 (0.232)	–	0.437 (0.087)

Variances of the parameters for each of the food treatments are shown on the diagonals (shaded), covariances below diagonal (in italics), and correlations above. All SEs are shown in brackets. V_A additive genetic variance, V_M maternal effects variance, V_B block variance (only fitted for female age at maturity due to lack of convergence for male size at maturity), $V_R$residual variance. Trait values were standardised to unit variance prior to analyses (see Methods for details).

male phenotypes. This is in contrast to estimates in many other taxa of strong cross-sex correlations in morphology (Kruuk et al. 2008; Poissant et al. 2010), and maturation time (e.g. Guntrip et al. et al. 1997). The differences between the sexes we observed here could potentially be due to the biology of mosquitofish. Females have indeterminate growth and their fecundity increases with body size (Bisazza et al. 1989; Callander et al. 2012), while males stop growing upon maturation. Selection pressures on growth and maturation rates are thus likely to be highly sex-specific. Sex differences in heritability of traits might be due to sexual selection acting more strongly on males, thereby depleting the amount of additive genetic variation expressed in males (Van Homrigh et al. 2007). Lower heritability of female traits has also been found in other species, for example for morphological traits in house sparrows (Jensen et al. 2003). However, the implications for evolutionary dynamics of differences in heritability between the sexes still remain relatively underexplored.

Within males, the relative importance of heritable genetic effects also differed among sexual traits. There was a low, non-significant heritability for relative gonopodium size (0.039 ± 0.083 SE; Table 1). Interestingly, this is similar to an estimate of realised heritability based on artificial selection on relative gonopodium length in the same study population (0.028 ± 0.006 SE, Booksmythe et al. 2016)—a reminder that a response to selection is possible even when heritability is low. Sperm number showed no evidence of additive genetic variance, but sperm velocity did. The low heritability of sperm number may indicate that sperm quantity is highly condition-dependent (e.g. influenced by diet; see O'Dea et al. 2014). Sperm velocity showed significant heritability in the 'top' model (Table 2), which could potentially fit with Y-linked effects as suggested in other poecilids (e.g. Evans 2011). We note however that the

estimate was lower and not significant in the basic model with maternal effects (Table 1), so there was no strong support for significant heritability of sperm velocity across all models.

Maternal effects contributed to trait variance for both males and females. Female mosquitofish invest in their offspring prior to fertilisation by provisioning eggs (lecithotrophy; Fernández-Delgado and Rossomanno 1997; Pollux et al. 2014) and also possibly via subsequent nutrient transfer to embryos (see Marsh-Matthews et al. 2005; Marsh-Matthews et al. 2010). Our results suggest that differences between mothers in their prenatal allocation of resources to either eggs or embryos have important implications for their offspring's subsequent development: maternal effects were still apparent in traits measured at sexual maturity (see also Kruuk et al. 2015). Further, in addition to the overall presence of maternal effects, we observed significant maternal-by-environmental variance (M × E) interactions for female age at maturity and male size at maturity. These interactions were apparent even though the food treatment was applied after maternal provisioning ended. They indicate that studies of maternal effects need to consider the potential impact of environmental heterogeneity: here, we would have reached a very different conclusion as to the importance of maternal effects had we only considered offspring reared under 'control' rather than 'low food' conditions. We found a significant positive covariance of the maternal effects across environments for female age at maturity. That is, mothers with maternal effects that caused their daughters to take longer to mature in the control environment also had daughters that took longer to mature in the low food environment (Fig. 2, Table 3). For males, however, there was no support for covariance across the food treatments for maternal effects on male size – probably due to the very low maternal effects variance expressed in the low food environment. We therefore found no evidence for trade-offs in maternal effects across environments. Similarly, Charmantier and Garant (2005) also find little evidence of genetic trade-offs via negative cross-environment genetic correlations, also suggesting that the role of environmental heterogeneity in generating life-history trade-offs remains unclear (see also discussion in Kruuk et al. 2008). As a final point, as noted in the Methods, our estimates of maternal effects variance could be inflated by dominance genetic variance and the separation of the two is a challenging issue for many studies (Wolak and Keller 2014). Data from different systems indicate that dominance genetic variance itself is typically small relative to additive genetic variance (Hill et al. 2008; Wolak and Keller 2014; Zhu et al. 2015). There is nevertheless the possibility that our estimates of M × E are inflated by dominance genetic-by-environment (D × E) interactions. There are examples of changes in non-additive genetic variance across environments in some taxa (e.g. Blows and Sokolowski 1995; Chirgwin et al. 2017; Kumar et al. 2015), but in general evidence for D × E appears to be markedly less prevalent than for G × E. We note also that, in turn, estimates of dominance variance in other systems may be inflated by maternal or shared environment effects if these are not properly modelled.

We found striking differences in our results from models incorporating maternal effects and maternal-by-environmental variances (M × E) compared to those without. Estimates of the variance due to additive genetic effects (V_A) were always higher in models where V_M was not estimated, indicating that ignoring maternal effects inflated estimates of V_A (Table S1). Similarly, when we compared estimates of $V_A \times$ Food (i.e. G × E) from models without and with $V_M \times$ Food (i.e. M × E), we found larger estimates if $V_M \times$ Food was not accounted for. For instance, for female age at maturity, $V_A \times$ Food was estimated at 0.054 ± 0.065 in the model with $V_A + V_M + V_A \times$ Food, but as $4 \times 10^{-8} \pm 6 \times 10^{-9}$ SE in the model with $V_A + V_M + V_A \times$ Food + $V_M \times$ Food. Similarly, for male size at maturity, the model of $V_A + V_M + V_A \times$ Food returned a G × E interaction estimate of 0.172 ± 0.078 SE, but the model $V_A + V_M + V_A \times$ Food + $V_M \times$ Food provided an estimate of $4 \times 10^{-7} \pm 4 \times 10^{-8}$ SE. The right-hand panels in Fig. 2 also show the much greater change in estimates of V_A across environments in models fitted without maternal effects. Although this is unsurprising, studies of genotype-by-environment interactions rarely also account for maternal-by-environment interactions. It is well established that the presence of maternal effects (or other non-additive causes of covariance between relatives, such as dominance variance) can inflate heritability estimates if not accounted for properly (Falconer and Mackay 1996; Kruuk and Hadfield 2007). In the same vein, our results indicate that estimates of G × E can be inflated by the existence of unaccounted-for M × E interactions. It is thus possible that previous studies of other populations have overestimated the role of G × E in driving phenotypic variation in systems where maternal effects (or other causes of increased similarity between relatives such as non-additive genetic effects) are important.

In sum, our study found sex differences in the genetic architecture underlying important phenotypic traits. Additionally, it suggests that maternal effects can shape phenotypic traits even when there is no postnatal investment and, furthermore, that differences between offspring of different mothers can interact with the offspring's rearing environment to influence their adult phenotypes. Our findings also illustrate the need to consider maternal-by-environmental interactions in quantitative genetic studies. G × Es have been well studied and there is increasing appreciation of their potential importance in evolutionary ecology (e.g. reviews by Des Marais et al. 2013; Hunt and Hosken 2014, respectively). Our

analysis here illustrates the need to also consider other potential contributors to environmental interactions when assessing G × E interactions, as failure to do so could result in an overestimation of the importance of G × E. Including M × E interactions may thus improve our understanding of the factors that contribute to phenotypic variance in different components of individuals' life histories.

Data archiving

Data available from Dryad: https://doi.org/10.5061/dryad.9s1gm

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Maternal-by-environment but not genotype-by-environment interactions in a fish without parental care

Supplementary Information

Sperm measurements

To strip ejaculates, males were anaesthetized in ice-cold water. Each male was then placed on a glass slide (coated with 1% polyvinyl alcohol solution (PVA) to prevent sperm bundles sticking to the slide) under a dissecting microscope. His gonopodium was swung forward and we applied gentle pressure to the abdomen to eject all the available sperm. We transferred the ejaculate to an Eppendorf tube with 100 - 900 µL of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, 10 mM Tris, pH 7.5). The amount of medium varied depending on the amount of ejaculate stripped to obtain accurate sperm counts. After the procedure each male was returned to his individual tank. Sperm collection was done blind to the treatment and family identity.

To estimate *sperm number*, we vortexed the sperm solution for one minute and then mixed it repeatedly with a pipette (20-30 times) to break up sperm bundles and distribute the sperm evenly throughout the sample. We placed 3 µl of solution on a 20 micron capillary slide (Leja) and counted the sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100× magnification. We counted five subsamples per sample from each male. See Vega-Trejo et al, 2016 for further details.

To estimate *sperm velocity* we analysed three samples per male. For each sample, we collected 3 µL of the diluted sperm following the same procedure used for sperm number. We collected the sperm velocity sample two days after we collected the number sample. We placed the 3 µL of diluted sperm in the centre of a cell of a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA) previously coated with 1% PVA. The sample was then activated with a 3 µL solution of 150 mM KCl and 2 mg ml-1 bovine serum albumin (Billard and Cosson 1992) and covered with a cover slip. We analysed sperm velocity within 30 seconds of activation for three subsamples to increase the number of velocity measures. We used an average of 109.3 ± 69.3 SD sperm tracks per ejaculate (minimum 10 sperm tracks / male). We recorded two standard measures of sperm velocity: (1) average path velocity (VAP): the average velocity over a smoothed

cell path and (2) curvilinear velocity (VCL): the actual velocity along the trajectory using a CEROS Sperm Tracker. Due to the near perfect correlation between VAP and VCL ($r=0.961$, $P<0.001$), we only use VAP in our analyses. See Vega-Trejo et al, 2016- for further details.

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Table S1. Comparison of models fitting additive genetic effects variance, maternal effects variance, and their interaction with food treatment. For each model, the log likelihood (log (L)) is presented. The difference in AIC value with the model with the lowest AIC value is presented (Δ AIC). Weight = Akaike model weight, relative to all 9 models for each trait (see Methods for definition). V_A = additive genetic variance, V_M = maternal effects variance, V_B = block variance, V_R = residual variance. Models are shown in descending order with respect to Δ AIC. Models in bold represent the models ranked within two AIC units of the top model.

Model	log (L)	AIC	Δ AIC	Weight	V_A ± SE	V_M ± SE	V_A × Food ± SE	V_M × Food ± SE	V_B ± SE	V_R ± SE
Female size at maturity										
V_A+V_M	**-417.145**	**842.291**	**0**	**0.456**	**0.310 ± 0.124**	**0.255 ± 0.060**			**0.056 ± 0.041**	**0.382 ± 0.066**
V_A+V_M+V_M×Food	**-416.872**	**843.745**	**1.454**	**0.221**	**0.321 ± 0.124**	**0.210 ± 0.078**		**0.045 ± 0.060**	**0.056 ± 0.042**	**0.368 ± 0.067**
V_A+V_M+V_A×Food	-417.145	844.291	2	0.168	0.310 ± 0.124	0.255 ± 0.060	3.862×10^{-8} ± 6.666×10^{-9}		0.056 ± 0.041	0.382 ± 0.066
V_A+V_M+V_A×Food+V_M×Food	-416.872	845.745	3.454	0.081	0.321 ± 0.124	0.210 ± 0.078	5.891×10^{-7} ± 1.074×10^{-7}	0.045 ± 0.060	0.056 ± 0.042	0.368 ± 0.067
V_M	-420.308	846.617	4.326	0.052		0.389 ± 0.048			0.088 ± 0.037	0.529 ± 0.031
V_M+V_M×Food	-420.210	848.420	6.129	0.021		0.364 ± 0.068		0.027 ± 0.058	0.088 ± 0.037	0.524 ± 0.032
V_A	-428.329	862.658	20.367	1.725×10^{-5}	0.760 ± 0.105				0.059 ± 0.049	0.239 ± 0.064
V_A+V_A×Food	-428.329	864.658	22.367	6.345×10^{-6}	0.760 ± 0.105		4.650×10^{-5}± 1.248×10^{-8}		0.059 ± 0.049	0.239 ± 0.064
Null	-489.612	983.223	140.932	1.138×10^{-31}					0.090 ± 0.033	0.895 ± 0.040
Model averaged estimates (across all models)					0.290 ± 0.138	0.243 ± 0.075	5.430×10^{-8} ± 8.791×10^{-8}	0.014 ± 0.031	0.056 ± 0.042	0.377 ± 0.073
Male size at maturity										
V_M+V_M×Food	**-466.625**	**941.249**	**0**	**0.574**		**3.813×10^{-8} ± 2.257×10^{-9}**		**0.311 ± 0.043**	**0.019 ± 0.015**	**0.603 ± 0.036**
V_A+V_M+V_M×Food	**-466.239**	**942.478**	**1.229**	**0.310**	**0.060 ± 0.074**	**4.989×10^{-7} ± 3.839×10^{-8}**		**0.284 ± 0.051**	**0.010 ± 0.018**	**0.574 ± 0.050**
V_A+V_M+V_A×Food+V_M×Food	-466.239	944.478	3.229	0.114	0.060 ± 0.074	2.674×10^{-7} ± 2.339×10^{-8}	4.068×10^{-7} ± 3.559×10^{-8}	0.284 ± 0.051	0.010 ± 0.018	0.574 ± 0.050
V_A+V_M+V_A×Food	-471.717	953.434	12.185	0.001	5.542×10^{-8} ± 5.675×10^{-9}	0.210 ± 0.048	0.172 ± 0.078		0.005 ± 0.016	0.547 ± 0.056
V_M	-475.727	957.454	16.205	1.738×10^{-4}		0.266 ± 0.042			0.018 ± 0.015	0.658 ± 0.036
V_A+V_M	-475.629	959.257	18.008	7.054×10^{-5}	0.038 ± 0.086	0.249 ± 0.056			0.014 ± 0.019	0.641 ± 0.055
V_A+V_A×Food	-480.662	969.323	28.074	4.599×10^{-7}	0.217 ± 0.112		0.286 ± 0.113		1.961×10^{7} ± 2.841×10^{8}	0.442 ± 0.063

V_A	-486.158	978.316	37.067	5.127×10^{-9}	0.449 ± 0.081				2.860×10^{7} ± 3.466×10^{8}	0.509 ± 0.062
Null	-513.820	1031.641	90.392	1.350×10^{-20}					0.039 ± 0.018	0.910 ± 0.040
Model averaged estimates (across all models)					0.026 ± 0.050	3.188×10^{-4} ± 6.618×10^{-4}	2.238×10^{-4} ± 4.691×10^{-4}	0.299 ± 0.049	0.015 ± 0.017	0.590 ± 0.044
Female age at maturity										
$V_A+V_M+V_M\times$Food	**-401.233**	**812.467**	**0**	**0.595**	**0.255 ± 0.112**	**0.162 ± 0.071**		**0.137 ± 0.063**	**0.044 ± 0.037**	**0.368 ± 0.062**
$V_A+V_M+V_A\times$Food+$V_M\times$Food	-401.233	814.467	2	0.219	0.255 ± 0.112	0.162 ± 0.071	3.719×10^{-8} ± 6.271×10^{-9}	0.137 ± 0.063	0.044 ± 0.037	0.368 ± 0.062
$V_M+V_M\times$Food	-404.289	816.578	4.111	0.076		0.263 ± 0.065		0.135 ± 0.064	0.088 ± 0.036	0.493 ± 0.031
V_A+V_M	-404.458	816.916	4.449	0.064	0.241 ± 0.110	0.256 ± 0.055			0.045 ± 0.036	0.414 ± 0.061
$V_A+V_M+V_A\times$Food	-404.077	818.153	5.686	0.035	0.207 ± 0.116	0.259 ± 0.056	0.054 ± 0.065		0.050 ± 0.038	0.393 ± 0.065
V_M	-407.291	820.582	8.115	0.010		0.353 ± 0.045			0.086 ± 0.035	0.532 ± 0.031
V_A	-421.267	848.535	36.068	8.765×10^{-9}	0.659 ± 0.099				0.020 ± 0.036	0.304 ± 0.063
$V_A+V_A\times$Food	-420.778	849.556	37.089	5.260×10^{-9}	0.622 ± 0.108		0.067 ± 0.069		0.022 ± 0.038	0.275 ± 0.067
Null	-472.685	949.370	136.903	1.114×10^{-30}					0.085 ± 0.031	0.873 ± 0.039
Model averaged estimates (across all models)					0.240 ± 0.119	0.177 ± 0.079	0.003 ± 0.009	0.116 ± 0.074	0.045 ± 0.038	0.373 ± 0.067
Male age at maturity										
V_M	**-436.383**	**878.765**	**0**	**0.4154**		**0.257 ± 0.257**			**0.023 ± 0.016**	**0.618 ± 0.035**
$V_M+V_M\times$Food	**-435.892**	**879.783**	**1.0181**	**0.2497**		**0.215 ± 0.061**		**0.060 ± 0.061**	**0.023 ± 0.016**	**0.602 ± 0.037**
V_A+V_M	-436.383	880.765	2	0.1528	3.800×10^{-8} ± 2.151×10^{-9}	0.257 ± 0.041			0.024 ± 0.016	0.618 ± 0.035
$V_A+V_M+V_M\times$Food	-435.889	881.778	3.0133	0.0921	0.005 ± 0.076	0.213 ± 0.070		0.060 ± 0.062	0.022 ± 0.019	0.599 ± 0.052
$V_A+V_M+V_A\times$Food	-436.383	882.765	4	0.0562	6.037×10^{-8} ± 3.417×10^{-9}	0.257 ± 0.041	2.310×10^{-7} ± 1.308×10^{-8}		0.024 ± 0.016	0.618 ± 0.035
$V_A+V_M+V_A\times$Food+$V_M\times$Food	-435.889	883.778	5.0133	0.0339	0.005 ± 0.076	0.213 ± 0.070	6.064×10^{-8} ± 5.275×10^{-9}	0.060 ± 0.062	0.022 ± 0.019	0.599 ± 0.052
V_A	-452.310	910.619	31.8541	5.028×10^{-8}	0.346 ± 0.346				0.003 ± 0.003	0.549 ± 0.062
$V_A+V_A\times$Food	-452.310	912.619	33.8541	1.850×10^{-8}	0.346 ± 0.079		1.819×10^{-7} ± 2.049×10^{-8}		0.003 ± 0.021	0.549 ± 0.062
Null	-469.740	943.480	64.7148	3.680×10^{-15}					0.036 ± 0.036	0.856 ± 0.038
Model averaged estimates (across all models)					4.620×10^{-4} ± 0.010	0.234 ± 0.142	1.299×10^{-8} ± 2.572×10^{-8}	0.020 ± 0.040	0.023 ± 0.016	0.591 ± 0.044

Relative gonopodium size										
V_M	**-501.979**	**1009.958**	**0**	**0.378**		**0.102 ± 0.035**			**0.012 ± 0.011**	**0.839 ± 0.045**
$V_A+V_M+V_A\times$Food	**-500.879**	**1011.757**	**1.799**	**0.154**	**0.019 ± 0.062**	**0.082 ± 0.042**	**0.083 ± 0.071**		**7.749×10^{-8} ± 5.965×10^{-9}**	**0.766 ± 0.059**
V_A+V_M	**-501.888**	**1011.776**	**1.818**	**0.152**	**0.037 ± 0.079**	**0.089 ± 0.045**			**0.005 ± 0.017**	**0.820 ± 0.060**
$V_M+V_M\times$Food	**-501.931**	**1011.861**	**1.903**	**0.146**		**0.094 ± 0.050**		**0.017 ± 0.059**	**0.012 ± 0.011**	**0.831 ± 0.050**
$V_A+V_M+V_M\times$Food	-501.840	1013.680	3.722	0.059	0.037 ± 0.080	0.081 ± 0.058		0.017 ± 0.058	0.005 ± 0.017	0.812 ± 0.064
$V_A+V_M+V_A\times$Food+$V_M\times$Food	-500.879	1013.757	3.799	0.057	0.019 ± 0.062	0.082 ± 0.042	0.083 ± 0.071	4.572×10^{-7} ± 3.519×10^{-8}	7.749×10^{-8} ± 5.965×10^{-9}	0.766 ± 0.059
V_A+V_AxFood	-503.490	1014.980	5.022	0.031	0.079 ± 0.063		0.096 ± 0.073		7.819×10^{-8} ± 6.435×10^{-9}	0.773 ± 0.064
V_A	-504.787	1015.574	5.616	0.023	0.120 ± 0.048				8.377×10^{-8} ± 5.661×10^{-9}	0.828 ± 0.056
Null	-508.285	1020.570	10.612	0.002					0.017 ± 0.011	0.937 ± 0.041
Model averaged estimates (across all models)					0.013 ± 0.043	0.083 ± 0.046	0.013 ± 0.034	0.003 ± 0.015	0.007 ± 0.012	0.750 ± 0.088
Male sperm number										
V_M	**-215.358**	**436.716**	**0**	**0.323**		**0.073 ± 0.051**			**0.085 ± 0.042**	**0.824 ± 0.068**
Null	**-216.726**	**437.452**	**0.736**	**0.224**					**0.099 ± 0.042**	**0.885 ± 0.062**
V_A+V_M	-215.358	438.716	2	0.119	8.342×10^{-7} ± 6.879×10^{-8}	0.073 ± 0.051			0.085 ± 0.042	0.824 ± 0.068
$V_M+V_M\times$Food	-215.358	438.716	2	0.119		0.073 ± 0.051		3.669×10^{-7} ± 3.026×10^{-9}	0.085 ± 0.042	0.824 ± 0.068
V_A	-216.726	439.452	2.736	0.082	7.762×10^{-8} ± 5.416×10^{-9}				0.099 ± 0.042	0.885 ± 0.062
$V_A+V_M+V_A\times$Food	-215.358	440.716	4	0.044	8.342×10^{-7} ± 6.879×10^{-8}	0.073 ± 0.051	8.342×10^{-7} ± 6.879×10^{-9}		0.085 ± 0.042	0.824 ± 0.068
$V_A+V_M+V_M\times$Food	-215.358	440.716	4	0.044	8.342×10^{-7} ± 6.879×10^{-8}	0.073 ± 0.051		8.324×10^{-7} ± 6.879×10^{-9}	0.085 ± 0.042	0.824 ± 0.068
V_A+V_AxFood	-216.726	441.452	4.736	0.030	2.800×10^{-7} ± 1.954×10^{-8}		8.959×10^{-7} ± 6.250×10^{-9}		0.099 ± 0.042	0.885 ± 0.062
$V_A+V_M+V_A\times$Food+$V_M\times$Food	-215.358	442.716	6	0.016	8.432×10^{-7} ± 6.879×10^{-8}	0.073 ± 0.051	8.342×10^{-7} ± 6.879×10^{-9}	1.138×10^{-7} ± 9.383×10^{-9}	0.085 ± 0.042	0.824 ± 0.068
Model averaged estimates (across all models)					1.247×10^{-7} ± 2.514×10^{-7}	0.042 ± 0.054	4.051×10^{-9} ± 1.104×10^{-8}	4.542×10^{-8} ± 7.846×10^{-8}	0.082 ± 0.044	0.774 ± 0.099
Male sperm velocity										

V_A	**-179.474**	**364.947**	**0**	**0.127**	**0.190 ± 0.085**				**7.034×10^{-8} ± 9.136×10^{-9}**	**0.695 ± 0.090**
V_A+V_M	**-179.458**	**366.915**	**1.968**	**0.115**	**0.175 ± 0.112**	**0.014 ± 0.075**			**7.057×10^{-8} ± 9.077×10^{-9}**	**0.697 ± 0.090**
V_A+V_AxFood	-179.474	366.947	2	0.114	0.190 ± 0.085		1.572×10^{-7} ± 2.042×10^{-8}		7.034×10^{-8} ± 9.136×10^{-9}	0.695 ± 0.090
V_M	-180.494	366.988	2.041	0.114		0.095 ± 0.062			0.025 ± 0.029	0.785 ± 0.072
Null	-181.831	367.663	2.716	0.110					0.043 ± 0.029	0.862 ± 0.064
$V_A+V_M+V_M\times$Food	-178.981	367.963	3.015	0.109	0.157 ± 0.089	4.982×10^{-7} ± 7.401×10^{-8}		0.087 ± 0.086	6.532×10^{-8} ± 9.714×10^{-9}	0.645 ± 0.096
$V_M+V_M\times$Food	-179.994	367.988	3.040	0.109		0.064 ± 0.074		0.096 ± 0.098	0.023 ± 0.029	0.724 ± 0.086
$V_A+V_M+V_A\times$Food	-179.458	368.915	3.968	0.104	0.175 ± 0.112	0.014 ± 0.075	1.857×10^{-7} ± 2.839×10^{-8}		7.057×10^{-8} ± 9.077×10^{-9}	0.697 ± 0.090
$V_A+V_M+V_A\times$Food+$V_M\times$Food	-178.981	369.963	5.015	0.098	0.157 ± 0.089	6.532×10^{-8} ± 9.714×10^{-9}	1.033×10^{-6} ± 1.536×10^{-7}	0.087 ± 0.086	6.523×10^{-8} ± 9.077×10^{-9}	0.645 ± 0.096
Model averaged estimates (across all models)					0.100 ± 0.115	0.014 ± 0.046	1.389×10^{-7} ± 1.928×10^{-7}	0.009 ± 0.044	0.008 ± 0.017	0.574 ± 0.172

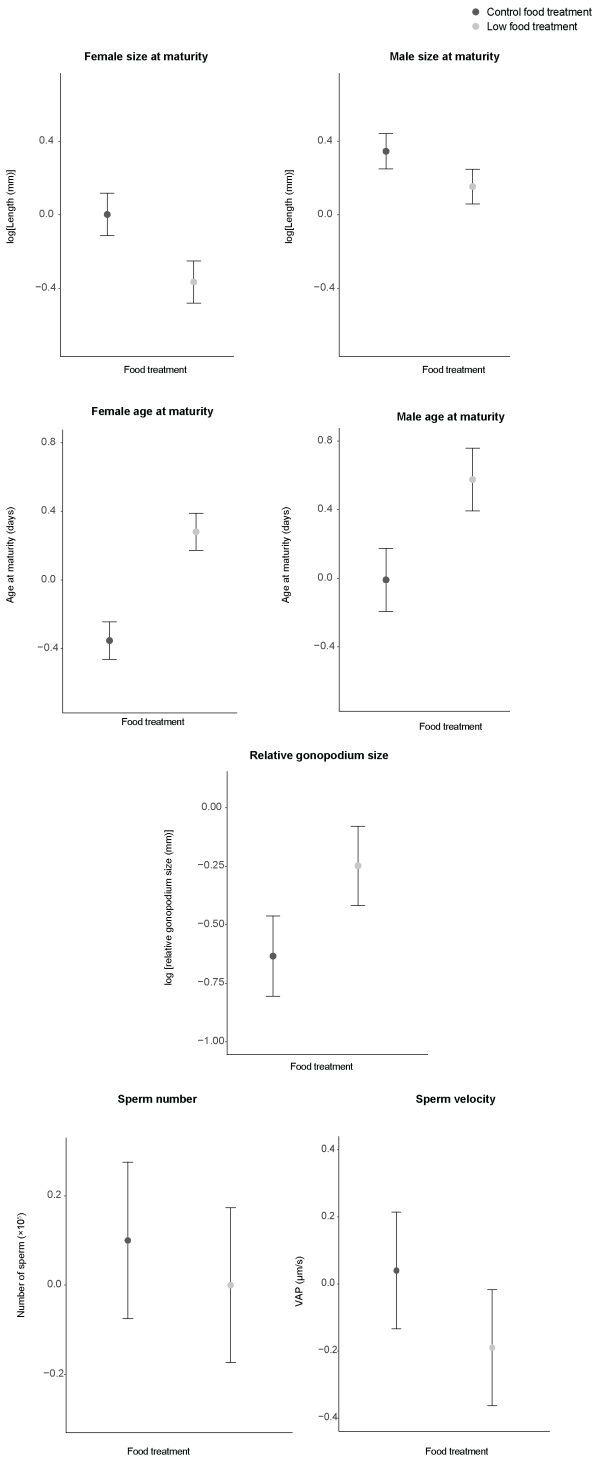
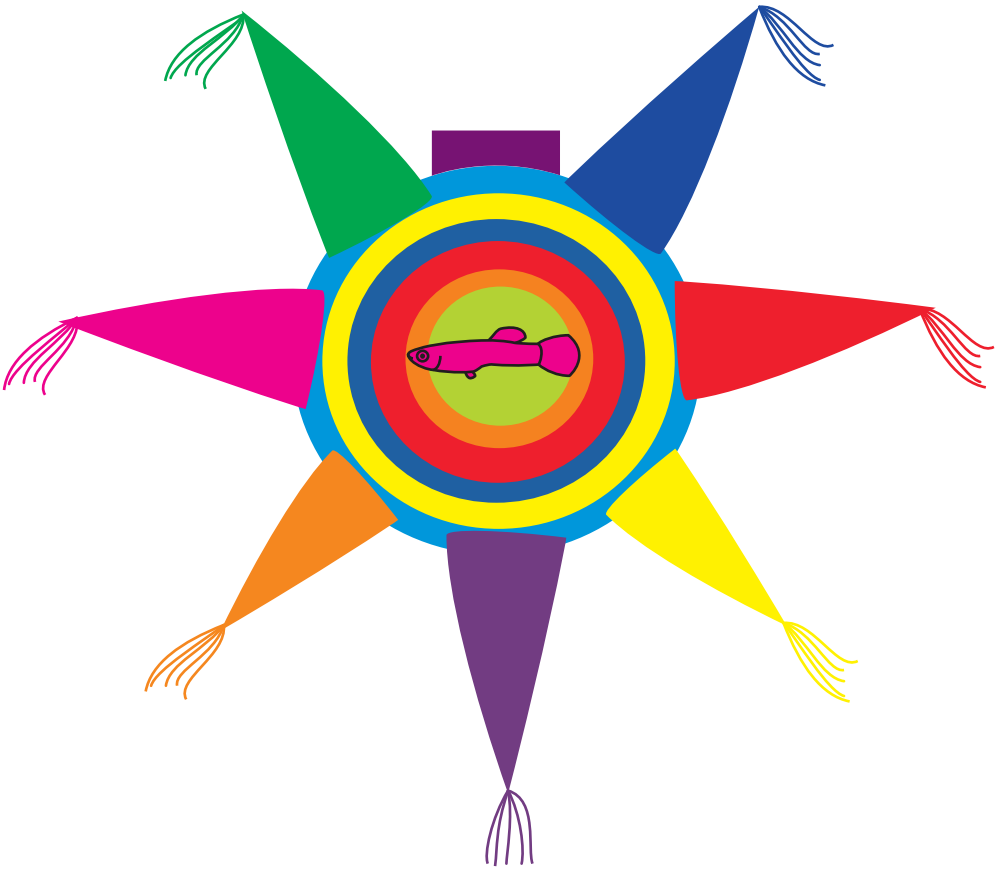


Figure S1. Parameter estimates ± SE for the effect of food treatment from a model with $V_A+V_M+V_B+V_R$ (see Methods for details). Values shown for the control treatment correspond to the intercept of the model. In all models, we fitted food treatment (control vs low food diet), inbreeding (inbred vs outbred), and generation (two levels, F_2-F_3). For sperm number and sperm velocity, generation was not included as a fixed effect because we only had data for F_2 males, but we included the age of the male at measurement (measured in days post maturity). Traits were standardized to unit variance and zero centred prior to analysis. Dark symbols represent values for fish in the control food treatment and light symbols represent values for fish in the low food treatment.

Synthesis and conclusions



Synthesis and conclusions

Given populations are likely to become more fragmented due to human interference, understanding how inbreeding depression acts, on which traits, at which stage in life, and how inbreeding interacts with the environment to affect fitness is of fundamental practical importance. Inbreeding depression is not universally higher in the wild or always greater in more stressed populations, and it affects traits differentially, making it hard to generalize. Although it is widely asserted that inbreeding depression reduces fitness, this pattern is not universal.

Mating behaviour is influenced by adaptive forces, which include direct benefits, additive genetic benefits, and non-additive genetic effects such as genetic compatibility and the avoidance of inbreeding depression (Tregenza and Wedell 2000; Kokko et al. 2003; Zajitschek et al. 2006). One way to avoid inbreeding is to recognise related individuals and avoid mating with them. Additionally, mating decisions can also be influenced by an individual's mating history and experience with previous mates (Jennions and Petrie 1997). Although recognition of familiar individuals can minimise the chance of offspring suffering from inbreeding depression (Pusey and Wolf 1996), I show that mosquitofish (*Gambusia holbrooki*) have a preference for novel mates only after mating with a previous mate had occurred (Chapter 1). In those scenarios, inbreeding avoidance is unlikely to be the key driver of preferences for novel partners. However, any costs associated with mating with kin might be outweighed by females biasing paternity through female cryptic choice or by males mating multiply.

One of the main costs of mating with relatives is a reduction in the number of offspring. However, the evidence for a negative effect of mating with a related male is mixed, with studies reporting fewer offspring or eggs (Pitcher et al. 2008; Johnson et al. 2010), but others finding no such effect (Simmons et al. 2006; Ala-Honkola et al. 2009). I add evidence from mosquitofish for a reduction in the number of offspring when females mate with a full sibling. Some of the potential explanations for females having fewer offspring when mating with related males are reduced fertilization success (i.e., low sperm survival due to sperm–female tract or egg interactions) and/or inbreeding depression lowering embryo survival (Pitcher et al. 2008; Johnson et al. 2010; Chapter 2).

The presence and magnitude of inbreeding depression may differ depending on which life stage and/or which traits are measured (Keller and Waller 2002). Additionally, it may only affect traits that affect fitness directly. For instance, I show that in *G. holbrooki*, the negative effects

of inbreeding depression may not be apparent in in growth rates, adult size, age at maturity, or sperm (Chapter 3, Chapter 4), but might still affect reproductive success (Chapter 5); illustrating the potential for hidden long-term costs of inbreeding depression. In general, inbreeding depression may only be detectable or magnified under certain conditions, such as when male-male competition is present. For example, inbreeding depression of male reproductive success is magnified in wild house mice under male-male competition (Meagher et al. 2000). Similarly, I show that inbred male mosquitofish have a lower reproductive success than outbred males when competing freely for females (Chapter 5).

The costs of a stressful environment early in life might only be expressed in the adult lifestage (Blount et al. 2003; Reichert et al. 2015). Individuals exposed to limited food availability during development can delay maturation to reduce the potential fitness costs of a smaller adult body size (Hector and Nakagawa 2012; Chapter 3). In turn, this could negatively influence long-term fitness benefits (Yearsley et al. 2004; Reichert et al. 2015). For example, female wood ducks that delay maturation have lower reproductive success (Oli et al. 2002). Similarly, male mosquitofish that delay maturation after being exposed to a low food environment early in life, show a reduction in sperm numbers and lower sperm velocity (Chapter 4). These effects show that the costs of a poor early environment might not be immediate, supporting the idea that costs can be delayed (Mugabo et al. 2010; Perez and Munch 2015), stressing the importance of looking at how different factors, such as nutritional constraints early in life and adult age, interact to determine adult performance (Chapter 4).

Variation in early nutrition due to differences in levels of parental care can clearly affect adult traits. For example, in dung beetles, developing larvae depend on nutrients provided by their parents which affects male body and horn size and thereby their mating success (Hunt and Simmons 2000). Even when there is no parental care, if parents experience a stressful environment this can still affect their offspring. For instance, male *G. holbrooki* that have been exposed to poor nutrition early in life have sons with smaller genitalia (Chapter 6), but this is not true for fathers reared on a control early diet. Parental effects can thus shape offspring phenotype in adult traits (e.g. Mousseau and Fox 1998; Pick et al. 2016), but their expression can also depend on the postnatal environment experienced by the offspring (Uller 2008; Badyaev and Uller 2009). In mosquitofish, I found that an interaction between maternal effects variance and the environment has the potential to affect traits expressed at maturity (Chapter 7). Although my results provide evidence for the role of parental effects in a species without parental care, they undoubtedly raise questions about the mechanisms generating these patterns, which are currently unknown and should be the focus of future studies.

The results of my thesis highlight the need to look at the interplay between the many different factors that can affect an individual's fitness. The complexity of forces shaping the evolution of key life-history traits might be influenced by trade-offs between life stages or traits. For example, inbreeding depression might not be evident based on the phenotype of individual traits, and only become apparent when looking at key fitness components. My thesis also highlights that variation in the early nutritional environment has effects on fitness that are potentially far reaching and can extend into adulthood.

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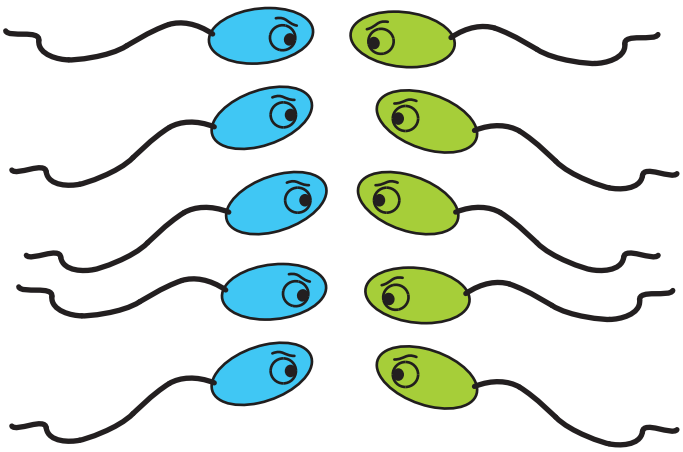
Appendix I

Why does inbreeding reduce male paternity? Effects on sexually selected traits

Evolution 71(11): 2728-2737



BRIEF COMMUNICATION

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Why does inbreeding reduce male paternity? Effects on sexually selected traits

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Mating with relatives has often been shown to negatively affect offspring fitness (inbreeding depression). There is considerable evidence for inbreeding depression due to effects on naturally selected traits, particularly those expressed early in life, but there is less evidence of it for sexually selected traits. This is surprising because sexually selected traits are expected to exhibit strong inbreeding depression. Here, we experimentally created inbred and outbred male mosquitofish (*Gambusia holbrooki*). Inbred males were the offspring of matings between full siblings. We then investigated how inbreeding influenced a number of sexually selected male traits, specifically: attractiveness, sperm number and velocity, as well as sperm competitiveness based on a male's share of paternity. We found no inbreeding depression for male attractiveness or sperm traits. There was, however, evidence that lower heterozygosity decreased paternity due to reduced sperm competitiveness. Our results add to the growing evidence that competitive interactions exacerbate the negative effects of the increased homozygosity that arises when there is inbreeding.

KEY WORDS: **Inbreeding depression, mate choice, paternity, sexual selection, sperm competition, poeciliid.**

Studies of wild animals often support the widespread expectation that being inbred reduces an individual's fitness (i.e., inbreeding depression) (reviewed in Hedrick and Kalinowski 2000; Keller and Waller 2002; Chapman et al. 2009). However, the contribution of sexually selected traits to lowering fitness is often unclear: relatively few studies have both experimentally manipulated the inbreeding status of males and ruled out potential confounding effects of natural selection on their reproductive success (but see, e.g., Drayton et al. 2010; Bolund et al. 2010; Zajitschek and Brooks 2010; Valtonen et al. 2014). At the within-population level, the comparative importance of sexual and natural selection in reducing the relative fitness of inbred and outbred males is unclear. There is, in addition, a potential link between lower relative fitness of inbred males and a population-level effect, but identifying this could depend on the causes of their reduced fitness. For example, sexual selection could exacerbate the detrimental effects of inbreeding in small populations. If inbred males are less likely to mate, even if they survive to adulthood, then the effective population size is smaller than that predicted based solely on survival to adulthood (Lynch and Walsh 1998).

There is a longstanding argument that male sexually selected traits will exhibit intense inbreeding depression (Brown 1997). This is partly because these traits, as with other major life-history traits, are important determinants of fitness, with a history of strong selection. Such traits are predicted to be more adversely affected by the bearer's inbreeding status because strong selection reduces the frequency of deleterious alleles that are dominant, and most deleterious alleles are therefore expected to be recessive (DeRose and Roff 1999; Roff and Emerson 2006). The resultant directional dominance means that alleles that lead to a beneficial increase (or decrease) in a trait are on average dominant over those that reduce (or increase) the trait (Wolak and Keller 2014). Inbreeding exposes these recessive mutations by increasing homozygosity. Consequently, sexually selected traits should show greater inbreeding depression than traits, such as minor morphological features that are weaker determinants of fitness so that directional dominance is weaker or absent (Lynch and Walsh 1998, p270; Cotton et al. 2004). Furthermore, sexually selected traits are often condition-dependent, and inbreeding status can adversely affect condition through its effects on the many loci

that influence resource acquisition (Rowe and Houle 1996). The expression of condition-dependent sexual traits should therefore "capture" and magnify the negative effects of inbreeding status on life-history traits that affect resource acquisition (Prokop et al. 2010). There is, indeed, evidence that sexually selected male traits favored during mate choice (e.g., de Boer et al. 2016) or fights (e.g., Sartori and Mantovani 2013) are negatively affected by the bearer's inbreeding status.

To date, there have been few attempts to compare the extent of inbreeding depression on different sexually selected traits. Sexual selection usually involves both pre- and postcopulatory phases (e.g., Devigili et al. 2015). Male reproductive success partly depends on the ability to inseminate females, generating precopulatory sexual selection for ornaments, displays, and weapons that function to attract or defend mates. But reproductive success also depends on the ability to convert insemination into fertilization, driving postcopulatory sexual selection for ejaculate traits and sperm performance (Parker and Pizzari 2010). The predicted effect of male inbreeding status on traits under precopulatory sexual selection is straightforward—it is expected to lead to reduced trait expression. The relationship between heterozygosity and sperm traits is more complex, however, because sperm are haploid. Inbreeding depression for sperm traits, such as motility, presumably arises through effects of male inbreeding status on associated diploid cells (Nayernia et al. 1996), for instance, germ cells that create sperm, and secretory cells that produce seminal substances that affect sperm performance. Losdat et al. (2014) recently reviewed the evidence that inbreeding negatively affects quantitative ejaculate measures (i.e., sperm count, ejaculate volume): inbreeding depression occurs in some species (e.g., Zajitschek and Brooks 2010; Maximini et al. 2011; Fox et al. 2012) but not others (e.g., Aurich et al. 2003; van Eldik et al. 2006). Losdat et al. also highlighted evidence for inbreeding depression in sperm morphology and motility in some species (e.g., Asa et al. 2007; Malo et al. 2010; Opatová et al. 2016), but, again, not in others (e.g., Okada et al. 2011; Ruiz-López et al. 2012; Gasparini et al. 2013).

Do these reported effects of inbreeding on sperm and ejaculate traits actually translate into lower fitness? And is there postcopulatory sexual selection against inbred males due to sperm competition (Losdat et al. 2014)? Interestingly, several studies that found no detectable differences in sperm quality or quantity between inbred and outbred males still reported that inbred males gain less paternity under sperm competition (e.g., *Tribolium castaneum*, Michalczyk et al. 2010; *Mesocricetus auratus*, Fritzsche et al. 2006). This suggests that despite no detectable effects of inbreeding status on ejaculate traits (either because measurable differences are small relative to measurement error, or because researchers are measuring the wrong traits), inbreeding depression can still occur due to sexual selection because of sperm competition. Despite this, few studies have used controlled experiments to investigate how inbreeding affects male fertilization success (Simmons 2011; Losdat et al. 2014).

To understand how inbreeding affects sexual selection requires studies that quantify its effects on different male traits and fitness components. Field studies of inbreeding depression are valuable but, strictly speaking, we have to manipulate heterozygosity levels experimentally, usually via controlled breeding designs, to identify causality (e.g., Slate and Pemberton 2006; see commentary in Opatová et al. 2016). Here, we do this using the mosquitofish, *Gambusia holbrooki*. In an earlier study, we showed that natural variation in heterozygosity predicts a male's share of paternity when wild-caught males compete for females in glasshouse ponds (Head et al., in press). In a second study, we experimentally created inbred (full-sib matings) and outbred males and again showed that inbred males had significantly lower reproductive success than outbred males when they freely competed for females (Vega-Trejo et al. 2017). In both experiments natural selection was effectively eliminated because males entered the mating pool as adults. This rules out potential effects of a male's inbreeding status on mortality affecting his success, as <2% of males died during the mating period. The lower success of inbred males is therefore, by definition, attributable to sexual selection: nonrandom variation in male reproductive success is due to competition for mates to gain insemination opportunities and sperm competition to fertilize eggs. This raises an obvious question: which component of sexual selection has a greater effect on male success: mating rate or sperm competitiveness?

We investigated several possible causes of the lower reproductive success of inbred male *G. holbrooki*. First, we quantified the effect of inbreeding status on precopulatory sexual selection, by examining male attractiveness to females. Second, we tested for postcopulatory sexual selection against inbred males due to inbreeding depression for ejaculate traits (sperm number) and sperm traits (swimming velocity). Finally, we use artificial insemination to test directly whether a male's inbreeding status lowers his fertilization success in a controlled setting. Our initial breeding design to generate inbred and outbred fish was set up to test whether inbreeding affects an individual's ability to compensate for a poor rearing environment (Vega-Trejo et al. 2016a). To this end, the sperm data presented here has been analyzed previously (Vega-Trejo et al. 2016b), albeit with a different question in mind (i.e., the effect of rearing environment, but not inbreeding). The data we present on male attractiveness and paternity was collected solely for the current study. Here, for simplicity, we only analyze the effects of inbreeding. In the Supporting Information, we show that rearing environment did not affect the focal traits, or share of paternity.

Methods

STUDY SPECIES

Gambusia holbrooki exhibits sexually coercive mating behavior whereby males consistently pursue and attempt to copulate with females. However, mate choice also occurs because females prefer to associate with large males that have a relatively long gonopodium (intromittent organ modified from the anal fin) (Kahn et al. 2009). Both of these male traits have previously been shown to predict insemination success (Head et al. 2015b). As for traits involved in postcopulatory sexual selection, evidence from another poeciliid, the guppy (*Poecilia reticulata*), suggests that sperm number and velocity are both predictors of fertilization success under sperm competition (Boschetto et al. 2011).

ORIGIN AND MAINTENANCE OF EXPERIMENTAL FISH

Our laboratory stock was collected in Canberra, Australia (mosquitofish were introduced to Australia in the 1920's; Ayres et al. 2010) from ponds that are less than 20 years old and likely to have been naturally colonized from a nearby artificial lake that was built in 1974. The mean heterozygosity for these wild fish is 0.27 (based on 3171 SNP loci: Head et al., in press). This is within the range of mean heterozygosity (based on SNP data) (0.23–0.63) reported for endemic mosquitofish in the southern United States (Vera et al. 2016).

The breeding design we used to experimentally create inbred and outbred fish has been fully detailed elsewhere (Vega-Trejo et al. 2015, 2016a) so we provide only a brief description here. We collected wild, gravid females and raised their offspring in single-sex tanks to ensure their virginity. All fish were kept on a 14-h light:10-hdark cycle at 28°C, and fed ad libitum with *Artemia* nauplii and commercial flakes. Once they had matured, males and females were randomly paired to create 58 full sibling families and the offspring from these families were then used in a fully balanced breeding design to create inbred and outbred experimental fish. We set up 29 blocks that each had mating individuals from two families (i.e., A and B). Brothers and sisters were paired to create inbred offspring (AA and BB), whereas males and females from opposite families were used in reciprocal pairings to create outbred offspring (AB and BA). Fish from these broods were individually reared in 1 L tanks on one of two diets, but including rearing diet in our analyses did not influence our main findings with respect to inbreeding depression (details in the Supporting Information). All data have been made available on Dryad (Marsh et al. 2017).

Experiment 1: Do females prefer to associate with outbred males?

We used two-choice tests to determine whether females spent more time with outbred than inbred males. We matched pairs of inbred and outbred males for size (tolerance < 1 mm) (inbreeding does not affect adult male size; Vega-Trejo et al. 2016a) and diet type. Trials took place in a 16 L tank (38 × 19 × 19 cm^3) divided into three sections: two end sections (5 × 19 × 19 cm^3), each housing a male, and a central section (28 × 19 × 19 cm^3) for the female (see Vega-Trejo et al. 2014). The sections were separated by a removable opaque screen (to minimize visual and olfactory contact before the trial) and by a mesh screen (made from tulle netting). To begin a trial, one male was randomly assigned to each end of the tank (but across all trials inbred and outbred males were at each end equally often) and a virgin stock female was placed in the central compartment. Fish were allowed to acclimate for 15min, after which we removed the opaque screens. Behavioral observations lasted 10 min during which we recorded female association time with each male (i.e., < 4 cm from a male's compartment). If a female did not visit the "association zone" of each male at least once ($N = 8$ of 117 trials), the same pair of males were then retested with a second female. We ran 109 successful trials. Our measure of association time predicts copulation attempts by male *G. holbrooki* in free swimming trials (Vega-Trejo et al. 2014). Data were collected blind to male inbreeding status.

Analysis

To test whether females preferred to associate with outbred males, we ran a generalized linear model (GLM) with quasibinomial error to account for overdispersion. We linked the amount of time the female spent with the outbred male and the amount of time spent with the inbred male and used this as the response variable in our binomial model. This can be broadly interpreted as the proportion of time spent with the outbred male, weighted by the total time spent with both males. Our key test is whether on the underlying latent scale the intercept differs from 0. This is equivalent to asking if the proportion of time a female spent with the outbred male is significantly greater than 50%, indicating a preference for outbred males.

Experiment 2: Do outbred males produce more sperm, or better performing sperm?

We collected sperm on three occasions. On the first occasion (Day 1), we stripped virgin males of sperm to measure their maximum sperm reserves. One day later (Day 2), we stripped males to estimate their sperm replenishment rate (i.e., sperm production in 24 h). Males do not fully replenish their sperm reserves until at least three days after being stripped (O'Dea et al. 2014), so using the number of sperm stripped on Day 2 is a valid measure of the replenishment rate. On the next day (Day 3), we stripped males to measure sperm velocity.

We stripped sperm following the methods of Matthews et al. (1997). Full details of our methods are in Vega-Trejo et al. (2016b), so we describe them only briefly here. Following

anesthetization in iced water, we placed males on their side under a dissecting microscope. We swung the gonopodium forward and at its base we applied gentle pressure to the abdomen so that the ejaculate was released into 100 μL of saline solution. We transferred the ejaculate to an Eppendorf tube with 100–900 μL of extender medium depending on the amount of ejaculate stripped. Males were then returned to their tanks.

To estimate the number of sperm, we thoroughly mixed the sample and then placed 3 μL of the solution on a 20 μm capillary slide (Leja) and counted the sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100 × magnification. We counted five subsamples per male.

To estimate sperm velocity, we analyzed three samples per ejaculate per male. For each sample, we collected 3 μL of diluted sperm (see above) and placed it in the center of a cell of a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA). The sample was then activated and covered with a cover slip. We analyzed sperm velocity within 30 sec of activation. We measured 109.3 ± 3.49 SE sperm tracks per ejaculate. We recorded two standard measures of sperm velocity: (1) average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path and (2) curvilinear velocity (VCL), the actual velocity along the trajectory. Due to a near perfect correlation of VAP with VCL ($r = 0.961$, $P < 0.001$), we only use VAP in our analyses.

We also measured male body size one week after the sperm extractions. Males were anesthetized by submersion in ice water, placed on their side, and photographed. We measured standard length (SL = snout tip to caudal fin base) in *Image J* (Abramoff et al. 2004). Data were collected blind to male inbreeding status.

Analysis

We measured maximum sperm reserves, sperm replenishment rate, and sperm velocity for 452 males (inbred = 224, outbred = 228). These data have been analyzed elsewhere with a focus on the hidden costs of compensatory growth (i.e., only analyzing the effect of diet; Vega-Trejo et al. 2016b). Here, we are interested in how inbreeding affects sperm traits.

To analyze the effect of inbreeding, we used generalized linear mixed models (GLMMs). We ran separate models for sperm at Day 1, Day 2 (i.e., replenishment rate) and sperm velocity (VAP). In each model, inbreeding status was a fixed effect and male standard length was a covariate to control for size-dependent variation in testes size, hence sperm reserves. Adult male size does not depend on his inbreeding status (Vega-Trejo et al. 2016a,b). In all GLMMs, we specified a Gaussian error structure and checked the distribution of model residuals to ensure this was appropriate. Gaussian error structure was chosen over Poisson errors (for count data) because the latter were highly overdispersed. Each model was fitted in *R* using the *lme4* package, with block, mother, father, and male ID as random factors. Model terms were tested for significance using the *Anova* function in the *car* package specifying Type III Wald chi-square tests so that the effect of inbreeding is estimated after controlling for body size.

We also calculated the inbreeding load (calculated as [Ln(Inbred mean/Outbred mean)]/0.25) following Losdat et al. (2014) to allow comparison of the effects of inbreeding status on male sperm traits with a previously published meta-analysis (Losdat et al. 2014). The inbreeding load was calculated for each block and then averaged across the 29 blocks. These are presented in Table 1.

Experiment 3: Do outbred males sire more offspring with sperm competition?

To determine whether outbred males have more competitive sperm, we artificially inseminated females with equal numbers of sperm from an outbred and an inbred male (matched as in Experiment 1). We then calculated their share of paternity.

To inseminate females, we first anesthetized each male in ice water, and stripped sperm as outlined in Experiment 2. We transferred 20 sperm bundles (in 3μL saline solution) from each male into microcentrifuge tubes that contained an additional 3 μL of saline solution, so that the tube contained 40 sperm bundles (20 per male). We then repeated this procedure to have two replicates per male pair. We allowed the sperm bundles to settle (ensuring we were able to collect all bundles) and used a micropipette

Table 1. The effect of inbreeding status on male sperm traits.

	Outbred			Inbred					
Trait	*N*	Mean	SE	*N*	Mean	SE	Mean Inbreeding Load	χ^2	P
Sperm number ($\times 10^{-5}$)	225	187.46	5.74	228	182.90	6.74	–0.024	0.094	0.759
Sperm replenishment ($\times 10^{-5}$)	225	56.41	3.01	228	55.47	2.82	–0.092	0.293	0.589
Sperm velocity	196	82.40	1.14	198	83.35	1.21	0.010	0.143	0.706

The inbreeding load following Losdat et al. (2014) (see Methods).

to draw 3 μL of the solution from the bottom of the tube. This solution, containing the intact sperm bundles from both males, was inserted into the reproductive tract of an anesthetized female. This process was carried out for both tubes so that each pair of males artificially inseminated two females. Males were preserved in ethanol for genotyping.

Following insemination, females were transferred into individual 1 L tanks containing a mesh divider and plastic plants to provide a refuge for offspring. Females were fed thrice daily with *Artemia* nauplii and checked twice daily for offspring. Offspring were collected, euthanized, and preserved in ethanol for genotyping. We continued checking for offspring for six weeks following insemination. In this time females could produce up to two broods.

To determine paternity, we took tissue samples from each male (124 males from 62 pairs) and all available fry ($n = 492$ offspring). For adults, DNA was extracted from the tail muscle/caudal fin. For offspring, DNA was extracted from the whole body (excluding head). DNA extraction and genotyping were performed by a commercial service (DiversityArray) using DArTseq (Elshire et al. 2011; Kilian et al. 2012; Courtois et al. 2013; Cruz et al. 2013; Raman et al. 2014). Full genotyping methodology details are in Booksmythe et al. (2016).

Genotyping was unsuccessful for only two of the 492 offspring, who were excluded from further analysis. After cleanup, our data yielded 2138 SNPs (average call rate 98.8%, average reproducibility rate 96.4%), which were used to calculate a Hamming Distance Matrix for the remaining 614 fish. Recent studies show that as few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals based on Hamming Distance values (Hu et al. 2015). In our study, we used a large number of high-quality SNPs to assign paternity between only two possible sires. The Hamming Distances between an offspring and its two possible fathers was compared, and the male with the highest genetic similarity (lowest distance) to an individual fry was assigned the paternity. We confidently assigned paternity to 488 fry but two fry appeared to be unrelated to either male: both had a Hamming distance similar to that between the focal fry and males from other test pairs (i.e., males that could not have been sires). These two fry were attributed to contamination during the insemination process, and excluded from analysis. We have elsewhere shown that assigning parentage at birth does not result in a downward bias in our estimate of inbred males' share of paternity due to greater embryo mortality of offspring sired by an inbred male (Vega-Trejo et al. 2017).

Using the SNP data, we also calculated the heterozygosity of each fish as the number of SNP markers that were scored as heterozygous divided by the total number of successfully classified markers for that fish (F_{het}). This is essentially a measure of genome wide heterozygosity, and F_{het} is identical to $1 - F_{hom}$, as used by Bérénos et al. (2016). Data were collected blind to male treatment.

Analysis

We analyzed the paternity data in two ways. First, we tested for an effect of inbreeding on the proportion of offspring sired by the outbred male using inbreeding status defined by our breeding design (i.e., a fixed categorical factor). We conducted this analysis because it was how we originally planned it, and to be comparable with analyses in our other experiments. We used a GLM with a quasibinomial error structure (to account for overdispersion). The proportion of offspring sired by the outbred male in each pair was created using the *cbind* function in *R* to weight each pair by the total number of offspring sired. Here, we pooled offspring from different broods (whether produced consecutively by the same mother and/or by both females) to avoid psuedoreplication. Because there were few pairs for which two females gave birth (8 out of 62) or for which females had multiple broods (2 out of 62), there was insufficient within-pair replication to justify a more complex model treating male pair as a random factor.

Second, we tested how relative heterozygosity influenced paternity. We did not originally intend to conduct this analysis because we were unaware this information would be available. We formally state this to distinguish between planned and exploratory data analysis (see Head et al. 2015a). In hindsight, however, this might be a better test for inbreeding depression because inbreeding is expected to reduce fitness *due to* lower heterozygosity (but see *Discussion*). Our breeding design created inbred and outbred males with significantly different mean heterozygosity (based on >2000 loci; see *Results*). Even so, the difference in heterozygosity between paired males varied (outbred–inbred difference: range –0.06 to 0.19; in 5 of 62 cases the outbred male was less heterozygous). We again used a GLM but with the weighted proportion of offspring sired by the more heterozygous male as our response variable and relative heterozygosity (high heterozygosity – low heterozygosity)/high heterozygosity) as the predictor. Using relative heterozygosity rather than the absolute difference in heterozygosity assumes that the effect of the difference in heterozygosity is nonlinear with respect to absolute heterozygosity. That is, the effect of a 0.1 difference in heterozygosity is expected to be greater for pairs with low absolute heterozygosity than it is for those with high absolute heterozygosity. Analyzing the data using the absolute difference in heterozygosity as the covariate gives qualitatively similar results (see *Results*).

Results

Based on data from over 2000 SNP loci, we found that inbred males had significantly lower mean heterozygosity than outbred males (mean ± SE – F_{het} inbred: 0.262 ± 0.005; F_{het} outbred:

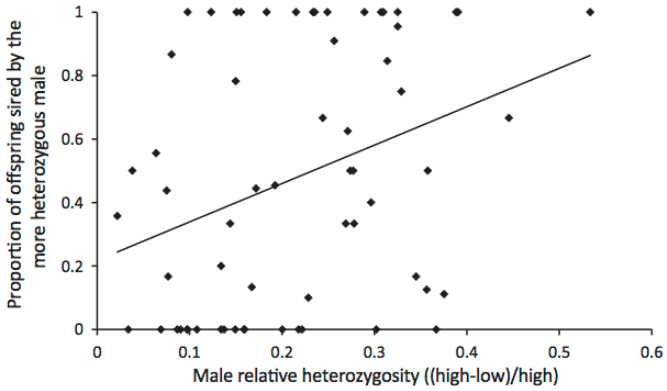

Figure 1. The relationship between male relative heterozygosity and the proportion of offspring sired by the more heterozygous male.

0.332 ± 0.005, $F_{(1,122)} = 99.99$, $P < 0.001$). The mean within-pair difference in heterozygosity of 20.1% is less than the 25% reduction expected from full-sibling matings in a fully outbred population (95% confidence intervals: 16.6–23.6%). This discrepancy might be attributable to early embryo mortality of more homozygous males (see Vega Trejo et al. 2015). It should also be noted that in another sample of inbred and outbred males from the same breeding design, the decline in heterozygosity was 23.2% (see Vega Trejo et al. 2017), which is closer to the expected 25% decline (see *Discussion*).

EXPERIMENT 1: DO FEMALES PREFER TO ASSOCIATE WITH OUTBRED MALES?

Females spent 45.5% (±2.0 SE) of the trial associating with a male, and they spent 48.7% (±3.2 SE) of their association time with the outbred male. This was not significantly different from 50% (intercept: $t = 0.41$, $P = 0.683$).

EXPERIMENT 2: DO OUTBRED MALES PRODUCE MORE SPERM, OR BETTER PERFORMING SPERM?

Outbred and inbred males did not differ significantly in their maximal number of sperm, sperm replenishment rate, or sperm velocity (Table 1). Larger males had significantly more sperm ($\chi^2 = 4.371$, $P = 0.037$) and significantly faster sperm ($\chi^2 = 4.070$, $p = 0.044$), but there was no relationship between male size and replenishment rate ($\chi^2 = 0.158$, $p = 0.691$).

EXPERIMENT 3: DO OUTBRED MALES SIRE MORE OFFSPRING WITH SPERM COMPETITION?

Outbred males sired 55.5% (±4.85) of offspring, which was not significantly different from 50% (intercept: $t = 1.128$, $P = 0.264$). However, a greater relative heterozygosity difference between paired males was associated with a significant increase in the share of paternity by the more heterozygous male ($t = 2.350$, $P = 0.022$) (Fig. 1). The same was true if we used the absolute difference in heterozygosity between the two males ($t = 2.437$, $P = 0.018$).

Discussion

It is widely accepted that mating with relatives can reduce the fitness of parents because they produce less fit offspring who suffer from inbreeding depression due to their increased homozygosity. However, the relative magnitude of the effect of being inbred on specific types of traits is often unclear. This is partly because nonexperimental studies of wild populations can rarely disentangle the potential causes of reduced fitness. For *G. Holbrooki*, we have previously shown that low heterozygosity (Head et al., in press) and inbreeding status (generated from controlled breeding) (Vega-Trejo et al. 2017) are associated with lower male reproductive success due to sexual selection. But it is not known why these males are less successful. In the present study, we quantified how inbreeding status affects male attractiveness, ejaculate traits, and share of paternity under sperm competition. To infer causality, we took an experimental approach to both the generation of variation in levels of inbreeding (i.e., used a breeding design) and how we measured male attractiveness and fertilization ability (i.e., two choice trials and competitive artificial inseminations).

ATTRACTIVENESS

Females in two-choice mating trials did not associate preferentially with outbred males. This contrasts with similar experiments in other species that have shown that inbred males are less attractive (e.g., in mice—Thom et al. 2008; Ilmonen et al. 2009; mealworm beetles—Pölkki et al. 2012; field crickets—Drayton et al. 2010; fruit flies—Okada et al. 2011; and butterflies—van Bergen et al. 2013). Other studies have, however, failed to

report inbreeding depression for male attractiveness (e.g., Michalczyk et al. 2010). Why do only some studies find female preferences for outbred males? The obvious explanation is that female mate choice decisions in some species are based on traits unaffected by reduced heterozygosity. Indeed, we have shown previously in *G. holbrooki* that inbreeding affected neither male body size, nor gonopodium length (Vega-Trejo et al. 2016a, b), two traits that influence female mate choice (Kahn et al. 2009) and male insemination success (Head et al. 2015b). However, even in the absence of direct effects of inbreeding status on sexual traits, there may still be benefits to females that avoid inbred males. Although it is often assumed that heterozygosity is not heritable (i.e., that mating with an inbred male will not lead to inbreeding depression in the resultant offspring), this is not always the case (Mitton et al. 1993; Nietlisbach et al. 2016). Heritability of heterozygosity occurs whenever allele frequencies are asymmetric (e.g., when allele frequencies are not 50:50 for biallelic loci; Mitton et al. 1993). That said, another explanation for the lack of a female preference for outbred males is simply that the study population (or its source population) has been sufficiently large over recent evolutionary time that the risk of inbreeding is low under random mating in most situations, so there is no meaningful selection on females for inbreeding avoidance.

SPERM TRAITS

We found no inbreeding depression for maximum sperm reserves, sperm replenishment rate, or sperm velocity. Losdat et al. (2014) reported that of 99 sperm traits examined in 24 species, a significant negative effect of inbreeding occurred in 48 cases, no detectable effect in 50 cases, and a significantly positive effect in one case. The general trend is therefore that inbreeding negatively affects sperm production and motility. The mean inbreeding load reported by Losdat et al. (2014) for sperm traits was –0.129 (95% CI: –0.209 to –0.049). How does this compare with our findings? In Table 1, we provide inbreeding loads for the sperm traits measured here—two of three are outside the 95% confidence intervals calculated from Losdat et al. (2014). Given our large sample sizes, it therefore seems likely that the lack of inbreeding that we report for these traits is a true null effect, and that our findings are anomalous with respect to the general pattern seen across many species.

Of course, sperm and ejaculate traits other than those we measured might be negatively affected by inbreeding. For example, many studies have shown that inbreeding creates sperm abnormalities (e.g., studies in Fitzpatrick and Evans 2009; see also Opatová et al. 2016). However, regardless of whether changes in sperm morphology or sperm count associated with inbreeding are detected, the evolutionary question is whether inbred males have lower fertilization success under sperm competition. It is necessary to formally test for this to determine whether postcopulatory sexual selection acts against inbred males.

SPERM COMPETITIVE ABILITY: PATERNITY

Our use of artificial insemination controls for any variation in the ability of males to inseminate females and potential differences in the number of sperm males transfer. The interpretation of our results depends on the exact statistical analysis. Based on our pedigree, outbred males sired more offspring than inbred males (mean share of paternity of 55.5%), but this was not statistically significant, implying that inbred males do not have less competitive ejaculates. However, because the use of SNPs provided data on the proportion of heterozygous loci (>2000 SNPs) for each male, we ran a post hoc exploratory test using this as another proxy for inbreeding (see discussion in Szulkin et al. 2010; Bérénos et al. 2014). Heterozygosity and inbreeding status are correlated in our sample ($r = 0.66$, $P < 0.001$). Even so, the share of paternity gained by the more heterozygous male increased with the magnitude of the relative (or absolute) difference in heterozygosity between the two competing males. This implies that heterozygosity, hence being more inbred, is under postcopulatory sexual selection because it reduces a male's competitive fertilization ability. Which of these two proxies provide a better estimate of an individual's overall level of heterozygosity is debatable. Mendelian sampling variance that affects the heterozygosity levels of individuals means that realized heterozygosity always varies around that expected based on the breeding design (i.e., a pedigree based inbreeding coefficient). This fact seemingly makes a SNP-based estimate more accurate, because it provides information about observed rather than average expected heterozygosity (see Visscher et al. 2006 for empirical data showing the effect of Mendelian sampling). The counterargument, albeit a Weak one, is that our SNP-based estimate will show sampling error, and might show a sampling bias, because it is based on a subset of markers, while the inbreeding coefficient is an unbiased genome-wide expectation of heterozygosity. One puzzle is why there was not a stronger correlation between the inbreeding coefficient (based on our experiment) and the observed level of SNP heterozygosity. There is clearly higher variation in homozygosity in the outbred population than expected given random mating in a single, large population. This is presumably due to recent variation in the extent of shared ancestry among wild individuals, which could arise due to, for example, periodic arrival of fish from small, isolated populations where inbreeding occurs more often. In general, future research testing how well different proxies of heterozygosity (e.g., pedigree-based inbreeding coefficients versus direct measures of heterozygosity) predict fitness would be useful (see Nietlisbach et al. 2017 for one such comparison).

Conclusions

Two previous studies showed that inbred (or less heterozygous) male *G. holbrooki* gain a smaller share of paternity when they compete for females, which we attribute to sexual selection against inbred males (Head et al., in press; Vega-Trejo et al. 2017). This could be due to a reduced ability to inseminate females and/or to lower ejaculate competitiveness. Here, we found no evidence that females are less likely to associate with inbred males. This suggests that inbred males are unlikely to have a lower mating rate due to reduced attractiveness, as occurs in some species (e.g., van Bergen et al. 2013). This does not, however, exclude other forms of precopulatory sexual selection. A male's mating rate in *G. holbrooki* also depends on his ability to: chase away rivals (e.g., Bisazza and Marin 1991), sneak up on females (e.g., Pilastro et al. 1997), and the success of the resultant insemination attempts (e.g., Head et al. 2015b). There was also no evidence that inbreeding affected sperm number or motility, despite our large sample size ($N > 400$ males). There was, however, evidence that less heterozygous (i.e., more inbred) males have less competitive sperm (Fig. 1). This implies that lower competitive fertilization ability might partly explain the lower paternity of inbred males in our earlier studies. If true, this raises the challenge of identifying which aspects of the ejaculate or of the sperm themselves are affected by inbreeding. Potential candidates include sperm longevity and the speed with which sperm penetrate an egg. Although our results suggest that postcopulatory sexual selection could be more important than mate choice in selecting against inbred male *G. holbrooki*, further studies on other components of precopulatory sexual selection, particularly in a competitive setting, are needed before general conclusions can be made about the relative role of pre- and postcopulatory sexual selection selecting against inbreeding.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Effects of inbreeding and diet on male sperm traits.

Appendix 2

Testing female preferences under more natural conditions: a case study on a fiddler crab

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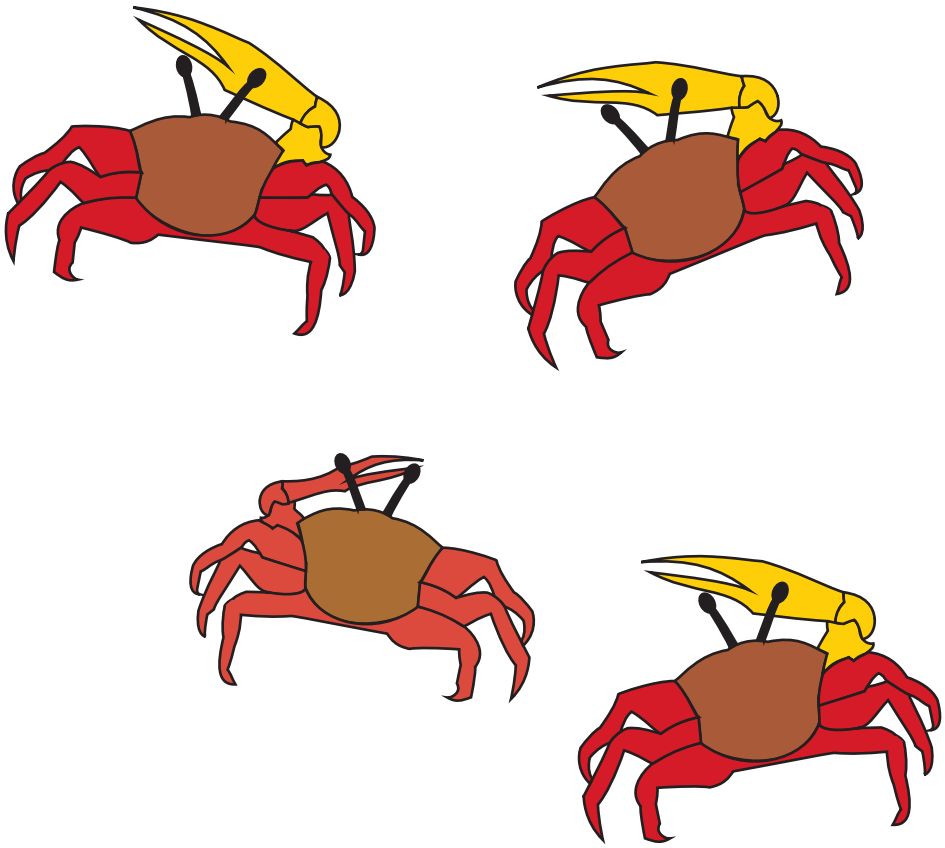

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ORIGINAL ARTICLE

Testing female preferences under more natural conditions: a case study on a fiddler crab

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Abstract
Mate choice is often affected by multiple factors, and there are often trade-offs associated with choosing a mate. Additionally, experiments that test for mate preferences usually rely on simple two-choice tests. These tests are, however, often less complex than the scenarios that individuals face in natural populations. Here, we test female choice in the fiddler crab *Uca mjoebergi*. We looked at female preference for wave rates and proximity to males in simple two-choice tests. We then mimicked a more natural choice scenario, where females faced a cluster of six courting males that differed in their distance from the female as well as in their wave rate. In addition, we tested whether female preferences under these more complex conditions were affected by the risk of predation. We found a preference for faster wave rates and closer males in two-choice tests. The preference for closer males was, however, only evident when the difference in distance was large (15 cm), not when it was small (3 cm). When females chose between six males, they preferred the males that waved faster, even if they were further away. We did not, however, find any difference in female choice when a simulated predator was present or absent. By examining a more realistic set of options that females face, we can paint a better picture of how females' trade-off costs and benefits during mate choice.

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Significance statement
Mate choice experiments often rely on two-choice tests. Mate choice, however, is often more complex under totally natural conditions. Using a two-choice experiment, we show that female fiddler crabs *U. mjoebergi* show a preference for faster wave rates and closer males. Under a more natural choice scenario, when choosing between six males, females preferred to travel longer to reach faster waving males. We found that female responses did not differ when a predator was present or absent. Designing choice experiments to more accurately mimic natural conditions will allow assessing trade-offs that occur in mate choice.

Keywords Two-choice · Predation · Sexual selection

Introduction

Mating preferences and preferred mate traits are often constrained by costs associated with mate choice. That is, females are often choosy when looking for potential mates and show a preference for particular sexual ornaments and signals (Jennions and Petrie 1997; Bonachea and Ryan 2011). However, there are costs associated with male sampling that can influence female mating preferences (Bakker and Milinski 1991; Cotton et al. 2006). This is especially true in species where females visit multiple males before selecting a mate. The costs of moving between potential mates are often high, and the benefits of finding a high-quality male must be weighed with the costs incurred by an increased sampling effort (Real 1990; Bonachea and Ryan 2011; Lindström and Lehtonen 2013). This trade-off between mate suitability and costs of sampling affects both sampling patterns and final mate choice (Backwell and Passmore 1996).

In the 1980s and 1990s, mate choice studies aimed to establish whether variation in mating success was non random, determine whether individuals vary in their mate choice, determine the range of trait values being chosen and figure out how individuals find the best potential mates (Real 1990; Andersson 1994; Jennions and Petrie 1997). To achieve these aims, relatively simple experimental designs involving mostly a two-choice design were employed. For example, two-choice phonotaxis experiments were used to show that female frogs had preferences for certain call variants, which were dependant on the surrounding environment and neighbours that a female was exposed to (Dyson and Passmore 1988; Backwell and Passmore 1990). Over the past 25 years, we have amassed a vast set of studies showing that mate choice is a complex process. Mate choice is plastic (Wacker et al. 2016), and it can involve multiple traits (Chenoweth and Blows 2006; Head et al. 2016), it often changes over time (Kahn et al. 2013), it can depend on voyeurism (Auld and Godin 2015) and it can even be irrational (Lea and Ryan 2015).

Although we understand many of the complexities of mate choice, we are lagging in our experimental designs for examining choices. Even in the early days of phonotaxis experiments in frogs, we knew that the preferences females exhibited in two-choice trials did not always translate into the natural setting (Telford et al. 1989), because the conditions of choice were more complex than those in a highly controlled two-choice test. This has recently become an issue that is again under discussion (Dougherty and Shuker 2015; Rowe and Arnqvist 2015): "we should take great care in designing studies of mate choice if our goal is to project our conclusions to natural populations" (Rowe and Arnqvist 2015).

Controlled experimental studies of mate choice are valuable as a supplement to correlational information from natural observations of male (or female) mating success, but we suggest that it is necessary to use experimental designs that more closely mimic the context in which mate choice naturally occurs (see Rowe and Arnqvist 2015). Studies that test female preferences under more realistic settings than two-choice trials could allow us to detect more subtle effects and interactions between factors. For instance, the costs of moving between potential mates are often high, and the benefits of finding a high-quality male must be weighed with the costs incurred by an increased sampling effort (Real 1990; Bonachea and Ryan 2011; Lindström and Lehtonen 2013). For example, female choosiness for quality song can decrease in female crickets when there is perceived predation risk (Atwell and Wagner 2015). Thus, more complex experimental settings could ultimately allow us to better translate experimental results into the natural setting and allow us to get a better grasp of trade-offs.

An ideal species to examine female mate choice under more natural conditions is the fiddler crab, *Uca mjoebergi*. We already know much about the natural behaviour of this species, and we have good information on their mate preferences in simple two-choice tests using robotic crabs (e.g. Booksmythe et al. 2008; Reaney 2009; Holman et al. 2014). Here, we examine female mating preferences under more complex conditions involving choice between six males at different distances from the female and either in the presence or absence of a predator.

Study system

U. mjoebergi is a small fiddler crab (<2 cm carapace width) that lives in high density (approximate density 37 ± 17 crabs/m^2; R. Slatyer, L. T. Reaney and P. R. Y. Backwell, unpublished data), mixed-sex populations on inter-tidal mudflats (Reaney and Backwell 2007). Each individual defends a territory with a central burrow that is a heat sink, a water source, a mating and incubation site and a place to escape from predators (Reaney and Backwell 2007). The burrow is surrounded by a small area (±10 cm diameter) that individuals use for feeding and courting. Mating occurs over 5–9 days in each 14–17 day tidal cycle (Reaney and Backwell 2007). Once a female is ready to mate she leaves her territory and wanders through the population of waving males, visiting an average of three males (briefly entering their burrows) before selecting a mate. Mating occurs in the male's burrow where he guards her for a few days until she releases her eggs onto her pleopods (Reaney and Backwell 2007). The male then leaves and seals the female into the burrow, where she remains for approximately 20 days to incubate her eggs. Females re-emerge at a nocturnal spring tide to release their pelagic larvae into the water (Reaney and Backwell 2007).

The natural mating behaviour of this species is complex. Female mating preferences vary both temporally and spatially. Females living in the high inter-tidal zone change their mating preference for male size over the 9-day mating period every semi-lunar cycle: early mating females select larger males with cooler burrows, slowing embryonic development; females mating later select smaller males with warmer burrows, accelerating development (Reaney and Backwell 2007; Milner et al. 2010). Females living lower in the inter-tidal zone, however, do not show this temporal variation: they select the same sized males throughout the mating period. It is only in the high inter-tidal zone, at the start of the fortnightly mating period, that large size confers a male mating advantage (Clark and Backwell 2015).

Females select mates from small clusters of courting males that surround her as she moves through the population, and they choose males with higher wave rates than those of nearby male competitors (Callander et al. 2012). Observations of natural mate-searching females also suggest that they are less selective (bypass fewer males) when the risk of predation is higher (Booksmythe et al. 2008).

From two-choice robotic crab trials, we know that females prefer males with larger claws (Reaney 2009; Milner et al.

2010) and males who wave at faster rates (Reaney et al. 2008; Reaney 2009). Females also prefer males that produce leading signals (Reaney et al. 2008), closer males (Booksmythe et al. 2008), have UV cues on their claws (Detto and Backwell 2009) and have the conspecific yellow claw colouration (Detto et al. 2006). Moreover, females avoid males that display from elevated positions (Holman et al. 2014), and female preferences are strongest when competing males are close to each other (Peso et al. 2014).

In this study, we aim to confirm that females prefer (i) faster wave rates and (ii) nearer males under simple two-choice experimental conditions. We then combine these two factors to create a more natural choice scenario where females are faced with a cluster of six courting males that differ in distance from the female as well as wave rate. We also test whether female preferences under these more complex conditions are affected by the risk of predation. Given that females prefer males that are closer and males with faster wave rates in two-choice trials, we predicted that females would have to trade-off males that were far with fast wave rates with males that were close with slow wave rates under predation risk.

Methods

We examined female mating preferences in a population of *U. mjoebergi* at East Point Reserve, Darwin, Australia (12.41° N, 130.83° E) in November 2015. The experimental test arena was set up in a clearing in the mangrove trees, on the edge of the study population. We tested female preferences using robotic crabs consisting of a twin-cam motor that moves a small metal arm in a waving motion, exactly mimicking the mate attraction wave. The motor is remotely controlled to regulate the exact timing of each wave using custom-designed software (see Reaney et al. 2008; Booksmythe et al. 2011; Holman et al. 2014 for further details of the robotic crabs). The motor was placed under the testing arena, and the metal arm protruded through the arena floor. The arm had a plaster replica of *U. mjoebergi* claw attached to it. For all trials, we used replicas of the same claw, each measuring 24 mm. The surface of the test arena was covered by a 1-cm-thick layer of mudflat sediment. We conducted two-choice tests on an arena that measured 40 × 60 cm and six-choice tests on an arena that measured 60 × 60 cm. For all experiments, the order and side of stimuli presentation was randomized. It was not possible to record data blind, because we focused on the female's response.

We captured mate-searching females, as they wandered through the population of courting males. These females are easily identified, as they approach and enter the burrows of waving males. We placed the captured females in the shade in a cup with a small amount of seawater. The females were released after they were tested, so that they could continue mate-searching. Females naturally visit numerous males before selecting a mate, so it is not unreasonable to test them in multiple trials. Before each trial, the female was measured (carapace width) and placed in a small, inverted, transparent cup on the arena surface. The robotic crabs were allowed to produce three full cycles of waving before the female was remotely released. A choice was scored when the female directly approached a claw and moved to within 2 cm of it. Trials were discarded if the female darted, ran to the edge of the area or remained stationary for >3 min. Each female was retested up to a maximum of three times and excluded if they were all non-responses. Females were tested in multiple trials but were only tested in the same type of trial once (i.e., two-choice vs six-choice). For the predation/no predation experiments, females were only tested in one of the trials to prevent past experience of predation affecting the trial outcome.

Two-choice trials (i) Fast vs slow wave rates, but same distance: we determined female preferences for fast over slow wave rates by giving the female a choice between two robots placed 5 cm apart and 20 cm directly in front of the female release point. One robot waved at 4.2 waves/min (slow rate), and the other waved at 16.8 waves/min (fast rate). (ii) Close vs far (small and large differences), but same wave rate: we determined preferences for closer over further males by giving the females a choice of two identical robots, both waving at the fast rate (16.8 waves/min), and both directly facing the female, but differing in their distances from the female release point. In one set of trials, one robot was 3 cm further from the female (14 vs 17 cm); and in another set of trials, one robot was 15 cm further away (14 vs 29 cm).

Six-choice trials We presented females with a choice of six robotic crabs, each one 3 cm further away than the next (14, 17, 20, 23, 26 and 29 cm). The two nearest robots both waved at the slow rate (4.2 waves/min). The middle two robots both waved at the medium wave rate (8.4 waves/min). The furthest two robots waved at the fast rate (16.8 waves/min). Figure 1 gives a graphical representation of the experimental arena.

We repeated the six-choice trials: once with a simulated predator and once without. The simulated predator consisted of a life-size model of a bird painted black and attached to a zip line that ran above the test arena (see Booksmythe et al. 2008 for a similar design). The bird was released at the same time as the female was released, and it flew at a decreasing height (20–10 cm) directly above the central line between the female release point and the robotic crabs.

Statistical analyses To determine female preference for (i) fast vs slow wave rates, but same distance (N = 20 trials) and (ii) close vs far (small and large differences), but same wave rate (N = 20 trials for small differences, N = 20 trials for large differences), we performed binomial tests.

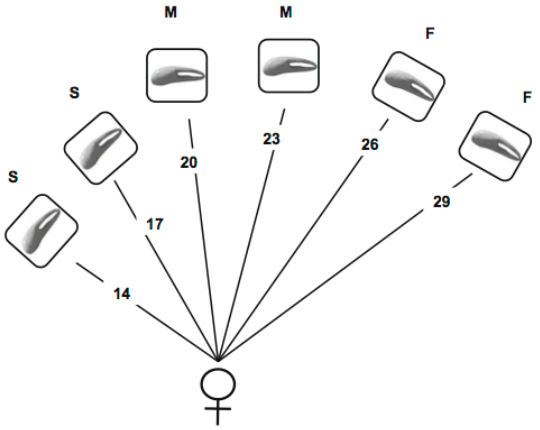

Fig. 1 Graphical representation of the experimental set up. Males were placed at 14, 17, 20, 23, 26 and 29 cm from the female release point. Wave rate increased with distance. *S* slow wave rate (4.2 waves/min), *M* medium wave rate (8.4 waves/min) and *F* fast wave rate (16.8 waves/min)

For our six-choice trials, we first compared whether female preference differed from a random distribution using a chi-squared test. We did this separately for each set of trials: without a predator (N = 40 trials) and with a predator (N = 40 trials). Since our two-choice trials revealed that small differences in distance between males did not influence female choice (see results below), we combined responses to pairs of robots with the same wave rates. We then tested whether they preferred certain wave rates using binomial tests. To control for multiple testing, we used false discovery rate correction (FDR; Benjamini and Hochberg 1995).

Using the combined responses of pairs of robots with the same wave rates, we tested whether female preference was different with or without a predator using a chi-squared test. To compare trials with and without a simulated predator for specific wave rate combinations, we performed a multinomial logistic regression using the package *nnet* using R version 3.2.4 (R Development Core Team 2012). We ran the multinomial regression twice, each time setting the reference category to a different choice option (i.e. near/slow and far/fast) to obtain p values for each of the possible preference combinations. This test looks at whether the risk of predation influences how females choose between the different wave rates. We additionally compared the number of times a female approached each of the wave rates across trials that differed in predation risk using binomial tests.

Results

Two-choice trials (i) Females showed a very strong preference for robotic crabs that waved at a faster rate (16.8 vs 4.2 waves/min) when they were both 20 cm away from the female release point. All 20 females approached the faster waving robot (binomial test $P < 0.001$).

(ii) When both robots waved at the same rate (16.8 waves/min), but were different distances from the release point, the females showed no preference for either signal when the difference in distance was small (14 and 17 cm away from the female, 13:7 responses, binomial $P = 0.26$). When the difference in distance was large, however, females preferentially approached the closer robot (14 vs 29 cm, 15:5, binomial $P = 0.04$).

Six-choice trials *without* a simulated predator when presented with an array of six robotic crabs, each 3 cm further away from the female release point and differing in wave rates, the number of responses was higher for far away males with a fast wave rate (Fig. 2a).

The number of responses to each of the six robots differed from a random distribution ($\chi^2 = 21.74$, $d.f. = 5$, $P < 0.001$). Combining the responses to pairs of robots with the same wave rates, we found a difference in female responses ($\chi^2 = 18.49$, $d.f. = 2$, $P < 0.001$) to the slow, medium and fast wave rates. They were more likely to approach the fast waving robots (over the medium or slow waving robots) even though they were further away (Fig. 2a). There was no difference in the number of approaches to the medium and slow waving robots. Binomial tests were slow vs fast $P_{FDR} < 0.006$; medium vs fast $P_{FDR} = 0.03$; slow vs medium $P_{FDR} = 0.22$.

Six-choice trials *with* a simulated predator The number of responses was higher for far away males with a fast wave rate (Fig. 2b). However, unlike the non-predator trials, the number of responses to each of the six robots did not differ from a random distribution ($\chi^2 = 7.98$, $d.f. = 5$, $P = 0.16$). Binomial tests were slow vs fast $P_{FDR} = 0.65$; medium vs fast $P_{FDR} = 0.04$; slow vs medium $P_{FDR} = 0.17$.

Comparison of trials with and without a simulated predator The presence of a simulated predator had no overall effect on the distribution of female responses across slow, medium and fast male wave rates ($\chi^2 = 6.15$, $P = 0.05$). There was also no effect of predation risk on the difference in female responses to specific combinations of wave rates (multinomial logistic regression: slow vs medium $P = 0.21$; medium vs fast $P = 0.99$; slow vs fast $P = 0.144$). Finally, we found no difference in the number of approaches to each of the wave rates when a predator was present or absent (binomial tests: slow-with predator vs slow-without predator $P = 0.27$; medium with predator vs medium without predator $P = 0.56$; fast with predator vs fast without predator $P = 0.56$).

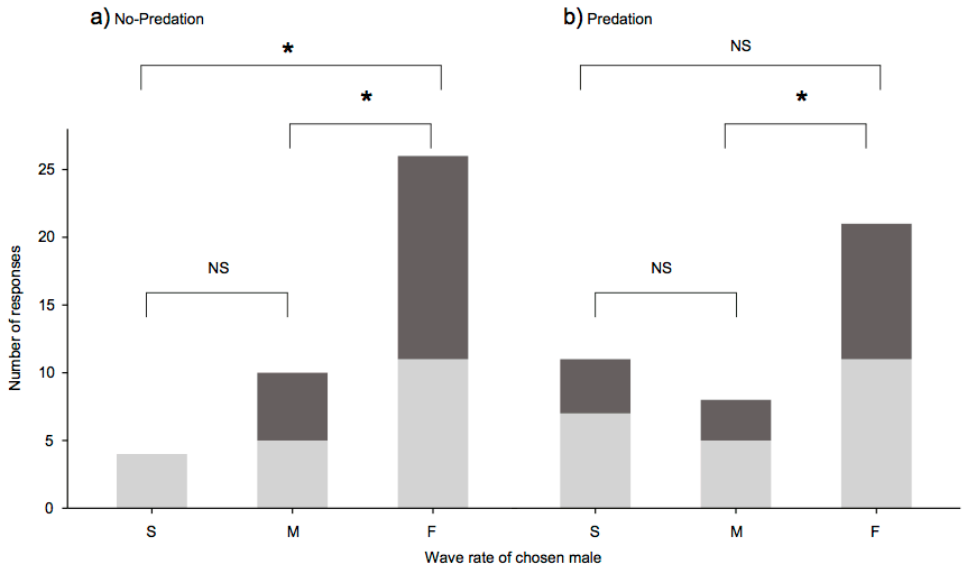

Fig. 2 Number of observations of females choosing either slow (*S*), medium (*M*) or fast (*F*) wave rates when there was no simulated predator (**a**) and when there was a simulated predator (**b**). Within each *bar*, the *lighter portion* is the number of responses to the nearer of the two stimuli and the *darker portion* is the number of responses to the further of the two stimuli

Discussion

The results from the two-choice trials are clear: (i) females strongly prefer males with a faster wave rate; (ii) females prefer closer males when the difference in distance is 15 cm; but (iii) they showed no preference for proximity when the more distant stimulus was only 3 cm further away. In the more complex six-choice trials, the females had to balance two variables: wave rate and distance. Under this scenario, females preferred far away males with fast wave rates over close males with slow wave rates. Based on the results from our two-choice trials, we suggest that females prefer males with a higher wave rate, in spite of having to travel a further distance.

Although our experimental design did not allow us to tease apart the effects of distance and wave rate, when the females were exposed to a risk of predation, females appeared to be less willing to travel long distances to reach a male with a high wave rate, although this effect is likely to be weak. When a simulated predator was present, female choice for males was no longer significantly different from random, but neither was it significantly different to female choice when there was no predator present. This is similar to a study in pipefish which found that the presence of a predator made mating random (Berglund 1993). In our study, females still approached the distant males with the fast wave rates more often than the closer males with the slower wave rates when a predator was present, but this choice was only marginally significant, and so we treat our observation with caution given its weak statistical support.

Females often need to weigh the benefits of choosing attractive mates with costs of predation that can be associated with mate-searching and assessment (e.g. Booksmythe et al. 2008; Atwell and Wagner 2015) or even with mating with certain males (e.g. Forsgren 1992; Johnson and Basolo 2003). When there is a risk of predation, females are expected to reduce their overall searching time by evaluating fewer males, assessing each male for less time, and discriminating less between males (Pomiankowski 1987; Real 1990; Hedrick and Dill 1993). Additionally, females can alter their mate preference (e.g. Gong and Gibson 1996; Evans et al. 2004; Kim et al. 2009; Zhu et al. 2012). Decreased investment in mate choice in the presence of a simulated predator may explain why female mate choice during our predation trials did not differ from a random distribution. Despite a shift in mate choice towards random mating, mate choice did not differ between the predation and no predation trials. This result might arise, because the degree to which predation can weaken female preferences is constrained between maximizing their choice (i.e. choice when no predators are present), and choosing at random, thus, effects might be expected to be small, and large sample sizes may be needed to detect the weakened preference.

Under natural conditions, a mate-searching female *U. mjoebergi* is more likely to approach a male whose wave rate is faster than his close neighbours (this study and Callander et al. 2012). Assessing the added effect of distance is difficult under natural conditions, since females do not always approach males in a straight line, not all males are

directly facing her, and males change their wave rates and relative proximity to the female as she moves towards them. We suggest that there is still a use for experimental choice trials, but that they would be even more useful if they better reflected some of the complexity found in the field.

The most common design for testing female preferences is the simple two-choice test in an experimental arena that controls for all but one variable (Wagner 1998; see Velásquez et al. 2015; Yasumiba et al. 2015; Zhu et al. 2016 for recent examples), but there are increasingly examples of more complex choice tests (e.g. Lea and Ryan 2015; Schwartz et al. 2016). There has been a recent call for a plurality of approaches including highly controlled two-choice trials, more natural test designs and experiments that assess mate choice in more natural social and environmental contexts (Dougherty and Shuker 2015; Rowe and Arnqvist 2015). For example, whether a female uses one or multiple cues to select a male might depend on the costs associated with the assessment of males (Fawcett and Johnstone 2003). More complicated experimental designs can not only demonstrate which cues are important in mate choice but can also help estimate the relative importance of indirect and direct benefits of mate choice (Chenoweth and Blows 2006; Owens 2006). By gaining insight into the effects of social and environmental variables on the strength of mate choice, we will better understand how signals evolve (Rowe and Arnqvist 2015).

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Compliance with ethical standards

Ethical approval This work was performed under an ethics approval permit (A2015/54) from the Australian National University Animal Experimentation Ethics Committee (ANUAEEC).

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Appendix 3

Male mate choice and insemination success under simultaneous versus sequential choice conditions

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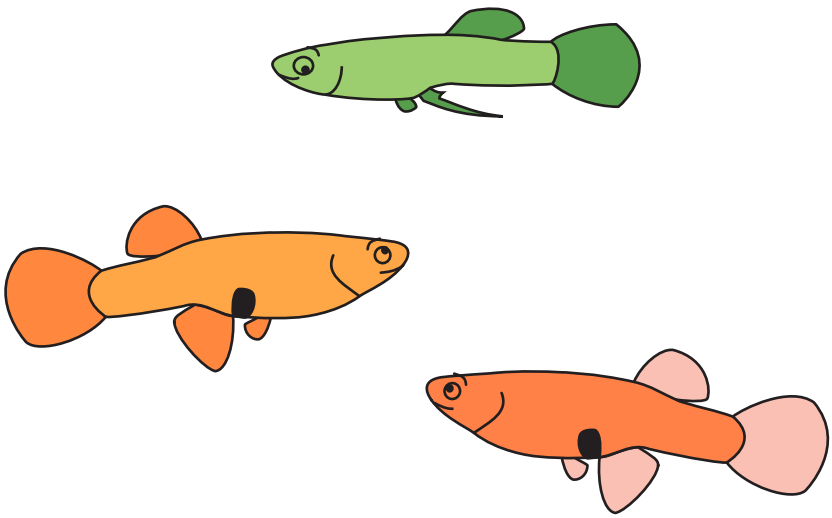

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Male mate choice and insemination success under simultaneous versus sequential choice conditions

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simultaneous choice
sperm allocation

Theory predicts that males should be choosier when encountering potential mates simultaneously rather than sequentially because there is no opportunity cost. Consequently, when mate encounter rates vary across space and time males might benefit from plasticity in mate preferences to match prevailing social conditions, preferring high-quality mates when females are encountered frequently and showing no preferences when females are encountered rarely. Here we investigated how encounter type (i.e. simultaneous or sequential) alters male mate preferences for female size in the mosquitofish, *Gambusia holbrooki*. We found that male mosquitofish attempted to mate with a relatively large female significantly more often than a relatively small female when presented with two females simultaneously. In contrast, males showed no such preference when sequentially presented with two females. Further, males attempted more copulations with absolutely larger females irrespective of encounter type. Despite these behavioural patterns, however, neither male insemination success nor the number of sperm transferred was influenced by female size or the encounter type. Our results provide support for the prediction that male mate choice is stronger during simultaneous choice encounters, but suggest that insemination success in *G. holbrooki* is partly under female control.

Male mate choice is more likely to evolve when there is variation in female quality, males have limited resources to invest in mating and there are low costs to being choosy (Bonduriansky, 2001; Edward & Chapman, 2011). To date, most empirical studies of male mate choice have focused on identifying the targets of choice (e.g. Pack et al., 2009; Tigreros, Mowery, & Lewis, 2014) and the benefits associated with choosing particular females (e.g. LeBas, Hockham, & Ritchie, 2003; Kekalainen, Huuskonen, Tuomaala, & Kortet, 2010; Nordeide, Kekäläinen, Janhunen, & Kortet, 2013). These studies have highlighted that male mate choice can evolve in a broad range of mating systems. There is, however, far less understanding of what contributes to variation in the presence and the strength of male mate choice among populations and between species (but see Dougherty & Shuker, 2014).

A key factor in the evolution of male mate choice is the relationship between the number of receptive females (i.e. mate encounter rate) and a male's capacity to mate. Male mate choice is predicted to evolve when mate availability is high and male capacity to mate repeatedly is low (Edward & Chapman, 2011). If females are frequently encountered, it is even possible that two or more potential mates are encountered simultaneously. This makes male mate choice more likely as individuals can choose between the immediately available mates at no cost (i.e. rejection does not lower the mating rate). Consequently, even small differences in the profitability of each mating favour the evolution of choice. In contrast, during sequential encounters, choosiness lowers a male's mating rate because some females are rejected (Barry & Kokko, 2010). Simultaneous availability of mates is a general cue that mate encounter rates are likely to be high.

It is expected that when mate availability/encounter rates vary across space and time individuals should adjust their level of choosiness to the perceived mate availability (Svensson, Lehtonen, & Wong, 2010). Under low mate availability, such that mates are only sequentially encountered, individuals should take advantage of a current mating opportunity. Under high mate availability, especially if this leads to simultaneous encounters with mates, individuals should be choosier. This prediction is best studied in experiments that compare male choice in different social contexts to control for effects of variation in male 'time out' on choosiness. For example, experimental studies on fiddler crabs (*Uca* spp.) have

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shown that males do not discriminate between heterospecifics and conspecifics during sequential encounters but do during simultaneous encounters (Booksmythe, Jennions, & Backwell, 2011). Likewise, male sticklebacks, *Gasterosteus aculeatus*, and male salamanders, *Desmognathus santeetlah*, preferred to court larger females, but only when females were presented simultaneously rather than sequentially (Rowland, 1982; Verrell, 1995). This trend is widespread. Interestingly, however, when examining all available studies greater choosiness during simultaneous choice is observed for females, but not for males, indicating that males across species may respond less consistently than females to variation in encounter rate (meta-analysis: Dougherty & Shuker, 2014). Male mate choice involves decisions not only about whether to mate, but also how to allocate resources to each mating (Parker, 1998; Parker & Pizzari, 2010). For example, males can vary how much sperm they transfer depending on a female's size, condition or mating history (meta-analysis: Kelly & Jennions, 2011). Consequently it can be informative to look not only at mating behaviour but also at insemination success and the number of sperm transferred to different females. How social environments influence male allocation of sperm to females of different quality has mostly been studied in the context of sperm competition (review: Wedell, Gage, & Parker, 2002). Theory predicts that males should adjust sperm allocation in response to the risk and intensity of competition (review: Parker & Pizzari, 2010; meta-analysis: Kelly & Jennions, 2011). Less is known about how males adjust sperm allocation to other social cues. More specifically, there are few studies designed to directly compare sperm allocation under different mate encounter scenarios (for a noteworthy exception see Cornwallis & Birkhead, 2006). However, greater sperm allocation to high-quality females has been shown for males exposed to females both simultaneously (e.g. two-choice tests: Cornwallis & Birkhead, 2006) and sequentially (e.g. 'no-choice' tests: Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2010; Rubolini et al., 2006; see also Appendix S2 of: Kelly & Jennions, 2011). These studies suggest that males can allocate sperm strategically, even during sequential mate choice.

Here we investigated how encounter type (i.e. simultaneous or sequential) affects male mate preferences for larger females in the mosquitofish, *Gambusia holbrooki*. Mosquitofish are well suited to investigating the causes of variation in male mate choice. First, they have internal fertilization and males transfer sperm to females via a modified anal fin called the gonopodium (Constanz, 1989). Males do not engage in courtship but perform coercive 'sneak' copulations in which they approach a female from behind and thrust their gonopodium towards her gonopore (Bisazza, 1993; Bisazza & Marin, 1995). This occurs repeatedly, which makes it possible to quantify male mating attempts (e.g. Booksmythe, Backwell, & Jennions, 2013). Second, female size varies considerably and is strongly correlated with fecundity (Bisazza, Marconato, & Marin, 1989; Callander, Backwell, & Jennions, 2012; Deaton, 2008). Thus, there are clear benefits to mating with larger females. Despite the likely benefits, however, male preferences for large females are not universal: some studies show a male preference for larger females (Bisazza et al., 1989; Callander et al., 2012; Mautz & Jennions, 2011), and others do not (McPeek, 1992). Furthermore, studies show that male preferences for large females can vary depending on other factors (e.g. trial type: Hoysak & Godin, 2007; mating history: Vega-Trejo, O'Dea, Jennions & Head, 2014). Third, males invest considerable effort trying to mate (attempting to copulate up to 20 times/min; Wilson, 2005) and may often make mate choice decisions when sperm stores are low (O'Dea, Jennions, & Head, 2014). Consequently, pursuing low-quality females could be costly in terms of lost opportunities to inseminate more profitable females. Finally, mosquitofish have highly dynamic social groups, forming mixed-sex shoals of varying size and sex ratio (Agrillo, Dadda, & Serena, 2008). The social environment varies widely, with the adult sex ratio and density of each sex changing throughout the breeding season (e.g. Kahn, Kokko, & Jennions, 2013). As such, males experience considerable variation in female encounter rates. Selection for plastic changes in mating behaviour given different mate encounter rates might therefore be strong.

Owing to the potential for individuals to encounter prospective mates simultaneously, studies of male mate choice in mosquitofish have only employed designs that use 'two-choice' (simultaneous) trials, measuring male association time with females presented behind dividers (e.g. Mautz & Jennions, 2011; Wong & McCarthy, 2009) and/or recording attempted sneak copulation rate in trials in which males can interact freely with two females (e.g. Hoysak & Godin, 2007; Vega-Trejo et al., 2014). To our knowledge there have been no experiments using 'no-choice' (i.e. sequential) mating trials to investigate male mate choice in *G. holbrooki*. It is therefore unknown whether males adjust their mate choice based on female encounter rate. These rate changes are exemplified at the extremes by simultaneous versus sequential encounters with receptive females.

In our experiment we independently manipulated mate encounter type and the relative size of the focal females encountered. We investigated how these two factors influenced male mate choice behaviour (number of attempted copulations) towards a focal female, insemination success (whether or not the female is inseminated) and sperm allocation (how many sperm are transferred). We predicted that (1) males will show a preference for relatively larger females and (2) if the mate encounter rate strongly influences the costs of choice then male preferences will be stronger during simultaneous than sequential trials. If the effects are weak, however, males should show a similar preference for relatively large females regardless of encounter type.

METHODS

Origin and Maintenance of Fish

Male fish were collected from two ponds (35°14′27″S, 149°5′27″E and 35°14′13″S, 149°5′55″E) in Canberra, Australia, in February 2014. The females used were first-generation laboratory-reared fish whose parents were collected from the same ponds in March 2013. Prior to the experiment all fish were housed in single-sex tanks at densities of 30–60 fish per 90 litres, and females were thus virgins. Fish were maintained at 27 °C on a 14:10 h light:dark cycle and fed *Artemia salina* nauplii and commercial fish flakes twice daily. Males were kept in the laboratory for 3–6 months prior to being used in our experiment.

Experimental Design

Each male was exposed to two females, one of which was the focal female. We independently manipulated (1) how males encountered the focal female (sequentially or simultaneously with the other female) and (2) the relative size of focal females (bigger or smaller than the other female). We then investigated the effects of these two factors and their interaction on male mate choice and sperm allocation using a 2 × 2 factorial design. Thus, we had four experimental treatments (sequential/relatively small female, sequential/relatively large female, simultaneous/relatively small female, simultaneous/relatively large female). Each male was assigned a unique pair of females and was only used once.

To manipulate the relative size of focal females we divided virgin females from our stock population into three size classes: small (<300 mg), medium (350–450 mg) and large (>500 mg). Female

weight was highly correlated with standard length measured from photographs taken after trials ($r = 0.938$, $P < 0.001$, $N = 288$). In our experimental trials the medium-sized female was always the focal female. Males in the relatively small focal female treatment were paired with a medium and a large female, whereas males in the relatively large focal female treatment were paired with a medium and a small female. Our manipulation of the focal female's relative rather than absolute size ensured that any difference in how males responded to a focal female was not confounded by her absolute size.

We also manipulated how females were presented to males to investigate whether male mate choice decisions depend on whether they encounter mates simultaneously or sequentially. In our simultaneous treatment both the focal female and the nonfocal female were placed in experimental tanks with the male on day 0 and remained with him for the duration of the experiment. In our sequential treatment only the nonfocal female was placed with the male on day 0. On day 6 immediately prior to behavioural observations we replaced the nonfocal female with the focal female.

Experimental Protocol

A schematic of the experimental protocol is given in Fig. 1. Prior to each experimental trial both sexes were anaesthetized in ice slurry. Excess water was removed from females which were then placed in a dish of water on a Mettler Toledo balance to measure wet weight (to the nearest mg). Males were stripped of sperm (details below) so that they all began the experiment with fully depleted sperm reserves.

Once fish had recovered, males were placed in one-half of a 7-litre aquarium (17 × 28 cm and 15 cm deep) that was divided in half by a mesh barrier. At the same time, depending on the treatment, the appropriate combination of females was placed in the other half of the tank. Focal females for the sequential choice treatment were kept separately in individual 1-litre tanks until needed for behavioural trials. On day 3 males were briefly taken out of their treatment tank and their ejaculates were again stripped. This allowed us to check whether male sperm number varied with the treatment a male experienced while replenishing sperm reserves. Previous studies have shown that the social conditions in which males are housed can influence sperm production (i.e. sperm priming: Aspbury & Gabor, 2004; Barrett, Evans, & Gasparini, 2014). If male sperm reserves differed between our treatments this could influence interpretation of our subsequent results. However, as there was no effect of treatment on sperm number at day 3 (quasi-Poisson generalized linear model, GLM: encounter type: $t_{1,112} = 0.446$, $P = 0.657$; relative size of focal female: $t_{1,112} = 0.302$, $P = 0.763$; interaction: $t_{1,112} = -1.605$, $P = 0.111$), we do not consider this further.

Behavioural Observations

After males had spent a further 3 days in their respective treatments (total of 6 days), we conducted behavioural observations of male mate choice. For our sequential choice treatment the nonfocal female was replaced with the focal female immediately before the behavioural observations. To begin an observation session we removed the mesh barrier and allowed males and females to interact freely. We allowed fish to acclimate for 2 min before recording their behaviour. We recorded the number of mating attempts towards the focal female (see Vega-Trejo et al., 2014). Behavioural observations lasted for 10 min and the fish were then allowed to interact for another 20 min. After 30 min we extracted sperm from the focal female's reproductive tract.

Collecting Sperm from Males

To ensure that male sperm number did not differ depending on our treatment (see above) sperm were stripped from males following the methods of Matthews, Evans, and Magurran (1997). Briefly, males were placed on their side on a glass slide under a dissecting microscope. The gonopodium was swung forwards and pressure was gently applied to the male's abdomen to expel sperm. Using a 10 µl pipette we transferred the stripped ejaculate to a microcentrifuge tube containing a known volume (100–300 µl) of saline solution (0.9% NaCl).

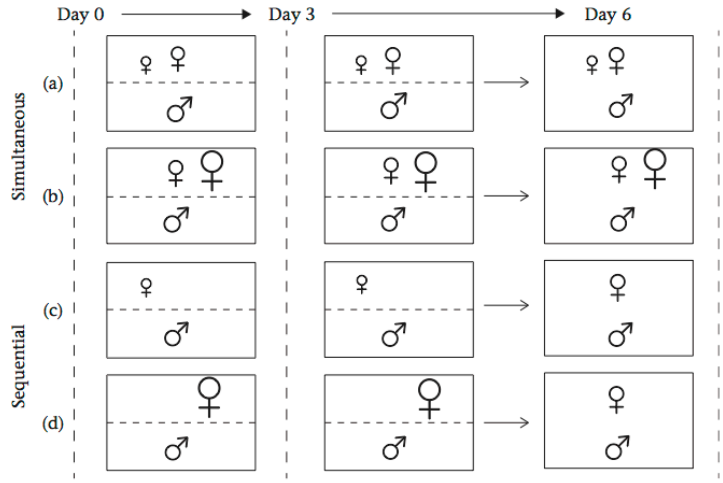

Figure 1. Schematic of the 2 × 2 experimental design. Dashed vertical lines indicate when males were stripped of sperm, dashed horizontal lines represent mesh barriers within tanks and green highlights the medium-sized focal females. (a) Simultaneous/relatively large female, (b) simultaneous/relatively small female, (c) sequential/relatively large female, (d) sequential/relatively small female.

Collecting Sperm from Females

We anaesthetized each female within 10 min of the behavioural trial and retrieved sperm from her gonoduct (see Pilastro & Bisazza, 1999; Pilastro, Giacomello, & Bisazza, 1997). A glass micropipette was used to flush the female's gonoduct with 30 µl of saline solution (0.9% NaCl).

Sperm Number

We counted sperm following the methods in Evans (2009). Briefly, sperm samples were vortexed for 1 min to break up sperm bundles and to evenly distribute sperm throughout the sample. Then 5 µl of the sample was placed on a Neubauer haemocytometer under x400 magnification (Kiyowa, Medilux-12 microscope). We photographed five cells of the haemocytometer so that sperm could later be counted blind to treatment. The five counts were averaged and the total number of sperm per male was then calculated by taking into account the concentration of the sample.

Ethical Note

This research was approved by the Australian National University Animal Ethics Committee (Approval no. A2011/64). We anaesthetized fish using an ice slurry prior to photographs and sperm collections because this method has been shown to be an ethical and effective method for anaesthetising small warm-water fish (Blessing, Marshall, & Balcombe, 2010). Further, it allows quick and easy handling of fish during these procedures because no special protection is needed for the experimenter. This reduces potential stress for fish arising from these procedures. Sperm were collected from each male ($N = 128$) a total of three times over a 7-day period during the experiment. Sperm were collected from females ($N = 192$) once during the experiment. All fish were monitored twice daily and stressed or dead fish were removed from the experiment ($N = 6$). The mortality rate of <2% observed during the experiment was comparable to normal laboratory mortality levels. After the experiment males were returned to stock tanks whereas females were euthanized in an overdose of clove oil (Cunha & Rosa, 2006).

Data Analysis

We used GLMs with appropriate error structures to investigate how mate encounter type and the focal female's relative size influenced (1) the number of copulation attempts with her, (2) whether a male inseminated the focal female or not and (3) how many sperm the male allocated to the focal female. All models included encounter type, the relative size of the focal female (categorical) and the absolute size of the focal female (continuous) as fixed effects. We also included the two-way interactions between encounter type, relative female size and absolute female size in the models. All GLMs were conducted using R v 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). The total sample size was 122 for the number of attempted copulations and insemination success (sequential/relatively large female: $N = 29$; sequential/relatively small female: $N = 29$; simultaneous/relatively large female: $N = 32$; simultaneous/relatively small female: $N = 32$). Sample sizes were lower than the number of trios set up ($N = 128$; 32 in each treatment) due to missing data and mortality. Our analysis of the number of sperm transferred to a female was restricted to trials in which the focal female received sperm (sequential/relatively large female: $N = 16$; sequential/relatively small female: $N = 16$; simultaneous/relatively large female: $N = 17$; simultaneous/relatively small female: $N = 17$).

RESULTS

Copulation Attempts

The number of copulation attempts directed towards the focal female differed depending on her relative size and the type of encounter (interaction: $t_{1,109} = -3.847$, $P < 0.001$). Males attempted to copulate significantly more often with a relatively larger than smaller focal female when females were presented simultaneously ($t_{1,56} = -3.532$, $P < 0.001$), but there was no effect of relative female size on the number of attempts when females were presented sequentially ($t_{1,56} = 1.165$, $P = 0.249$; Fig. 2a). The number of

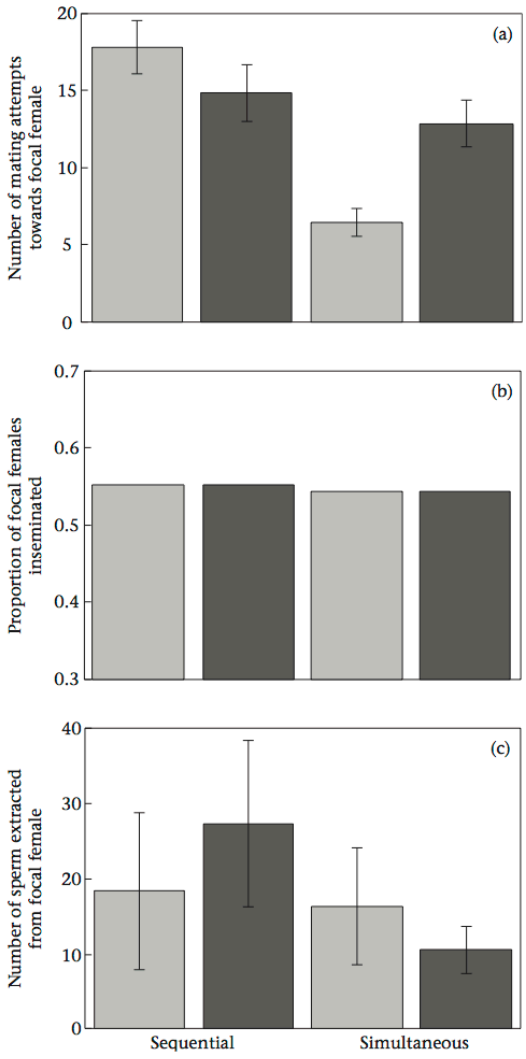

Figure 2. The effect of mate encounter type (sequential versus simultaneous) and the focal female's relative size on (a) the number of mating attempts a male directed towards the focal female (mean ± SE), (b) whether the focal female was inseminated or not and (c) the number of sperm allocated to the focal female (mean ± SE). Light bars represent relatively small focal females and dark bars relatively large focal females.

copulations directed towards the focal female also depended on her absolute size ($t_{1,109} = 2.180$, $P = 0.031$) and this effect was not mediated by the type of encounter (interaction: $t_{1,109} = -0.339$, $P = 0.736$) or the relative size of the focal female (interaction: $t_{1,109} = -0.877$, $P = 0.382$). There was no main effect of female relative size or encounter type on the number of copulation attempts with the focal female (relative size: $t_{1,109} = 1.013$, $P = 0.313$; encounter type: $t_{1,109} = 0.290$, $P = 0.772$).

Insemination Success and Sperm Transfer

Neither the focal female's relative size nor her absolute size influenced the likelihood of insemination (relative size: $t_{1,109} = -1.467$, $P = 0.145$; absolute size: $t_{1,109} = 0.565$, $P = 0.573$) or, for females that were inseminated, the mean number of sperm recovered from the reproductive tract (relative size: $t_{1,59} = -0.061$, $P = 0.952$; absolute size: $t_{1,59} = -0.152$, $P = 0.880$). Encounter type also had no effect on the likelihood that the focal female was inseminated ($t_{1,109} = 1.019$, $P = 0.310$) or, if she was, the mean number of sperm recovered from her reproductive tract ($t_{1,59} = -0.714$, $P = 0.478$). There was no interaction between the focal females' relative size and encounter type for either insemination success ($t_{1,110} = -0.132$, $P = 0.895$) or the number of sperm recovered ($t_{1,59} = 0.795$, $P = 0.429$; Fig. 2b, c). There was no interaction between the focal females' absolute size and encounter type for either insemination success ($t_{1,109} = -1.002$, $P = 0.318$) or the number of sperm recovered ($t_{1,59} = 0.591$, $P = 0.557$). There was also no interaction between the focal females' relative size and their absolute size for either insemination success ($t_{1,109} = 1.508$, $P = 0.135$) or the number of sperm recovered ($t_{1,59} = 0.000$, $P = 1.000$).

DISCUSSION

Males can afford to be choosier when their encounter rate with potential mates is high. This is because mate rejection then has a smaller effect on their actual mating rate (Jennions & Kokko, 2014). The simultaneous presence of potential mates is indicative of a high mate encounter rate, so one might predict that males are less choosy when they encounter potential mates sequentially (and with a long interval between encounters). In addition, choice between simultaneously available mates carries no opportunity cost (Barry & Kokko, 2010). If mate encounter rates vary then selection might favour male phenotypic plasticity in mating preferences based on cues that predict the prevailing social conditions (Svensson et al., 2010). Specifically, males should prefer high-quality females when females are frequently encountered (e.g. simultaneously), but show no preferences when females are rarely encountered. Our results support this prediction. Male *G. holbrooki* attempted to mate with relatively large females more often than relatively small females when presented with two females simultaneously. This agrees with several earlier studies on *G. holbrooki* (e.g. Bisazza et al., 1989; Callander et al., 2012; Mautz & Jennions, 2011; Wong & McCarthy, 2009; but see McPeek, 1992). In contrast, males showed no such preference when presented with two females sequentially. Furthermore, our results show that males directed more copulations towards absolutely larger focal females (even within the restricted 'medium' size range used here) and that unlike the case for relative female size this pattern existed whether females were encountered sequentially or simultaneously. However, despite males directing more copulation attempts at relatively larger females in simultaneous encounter trials and absolutely larger females overall this increased neither insemination success nor the number of sperm transferred.

Our experiment monitoring male mating attempts supports the claim that males are less choosy when females are encountered sequentially rather than simultaneously (Fig. 2a), although this is not a general trend across taxa (meta-analysis: Dougherty & Shuker, 2014). We cannot currently identify the proximate cause of this result. The encounter treatments should have generated a difference in perceived mate availability (and hence the perceived costs of rejecting the current mate), but they could also have affected a male's ability to discriminate between females. In a simultaneous encounter, males can directly compare the size of females, but cannot do so when females are encountered sequentially. Thus, both perceived mate availability and changes in discrimination ability could explain why a preference for relatively larger females was only seen in simultaneous encounters. Teasing apart these two effects is difficult, but possible. For example, Jordan and Brooks (2012) initially manipulated both how males encountered potential mates and the level of variation in female size in guppies, *Poecilia reticulata*. They then quantified male choice during sequential encounters. Males had stronger preferences for larger females if they had previously experienced greater variation in female size. Crucially, however, this effect was stronger for males that had previously encountered females simultaneously rather than sequentially (i.e. there was an interaction). This suggests that male choice is modified by perception of mate availability, but that discrimination between females based on size is reduced if mates are encountered sequentially (Jordan & Brooks, 2012).

There are at least two other potential explanations for the difference in male mating preferences between encounter types that we observed. First, during sequential encounters males had not seen the focal female before, whereas they had in the simultaneous encounters. In many species males prefer unfamiliar females (e.g. Kelley, Graves, & Magurran, 1999; LaDage & Ferkin, 2006). This does not appear to be the case in mosquitofish, however, as males do not prefer novel females unless they have previously mated with them (Vega-Trejo et al., 2014). We suggest that a preference for unfamiliar females is unlikely to explain our results. Second, in the simultaneous encounters there was an opportunity for female–female interactions to affect male mating behaviour that was absent in the sequential encounters. Some studies suggest that male mate choice might be more strongly related to female dominance than female size or that larger females might restrict male access to small females (Chen, Beekman, & Ward, 2011). Female–female interactions could explain why a mating preference for relatively larger females was only seen in the simultaneous encounters. We suggest that this is unlikely, however, as we did not observe any overtly aggressive interactions between females during mating trials.

We predicted that a male preference for relatively larger females would be weaker in sequential encounters, but the complete absence of a detectable preference is surprising. Previous research has shown that during sequential encounters females choose males based on their attractiveness relative to those that they have previously encountered and often 'trade up' (e.g. Kozak, Head, Lackey, & Boughman, 2013; Rebar, Zuk, & Bailey, 2011). Similar trends have been seen for male choice during sequential encounters (e.g. Wong, Jennions, & Keogh, 2004), but far less is known about how recent experience and relative female attractiveness influence male mate choice (but see Barrett et al., 2014; Jordan & Brooks, 2012; Svensson et al., 2010). In *G. holbrooki* previous studies demonstrating male choice for larger females have all been based on simultaneous choice trials (e.g. Callander et al., 2012; Mautz & Jennions, 2011; Wong & McCarthy, 2009). However, male choice for larger females has been shown in 'no-choice' trials in other poeciliid species (e.g. *P. reticulata*: Ojanguren & Magurran, 2004). This is a timely reminder of the dangers of extrapolating from simultaneous choice

experiments to choice in the field where mates are often encountered sequentially (Wagner, 1998).

It is intriguing that male attempted copulation rate increased with the absolute size of focal females, irrespective of whether encounters were sequential or simultaneous, but only for relatively larger females when encounters were simultaneous. One explanation for the increased attempted copulation rate towards absolutely larger females is that larger females may be easier to approach due to their decreased manoeuvrability (Pilastro et al., 1997). However, that there were more attempted copulations towards relatively larger females only during simultaneous encounters suggests that males have an additional 'active' preference for larger females, but that, as noted above, this preference is modified by either the costs of choice or the ease of detecting size differences between females.

There was no evidence for greater sperm allocation to relatively or absolutely larger females in either simultaneous or sequential encounter trials (Fig. 2). This suggests that *G. holbrooki* males do not strategically allocate sperm based on female size. In mosquitofish, males do not transfer all of their sperm reserves in a single mating (Evans & Pilastro, 2011). Thus, the opportunity cost of mating with smaller, less fecund females might be low. During simultaneous encounters males were only presented with two females so they might have been able to allocate the optimal (hence equal) amount of sperm to both females because they did not exceed their sperm reserves. Furthermore, rival males were always absent, which should reduce the propensity for males to increase sperm allocation beyond the minimum necessary to ensure fertilization (meta-analysis: Kelly & Jennions, 2011).

An alternative explanation invokes sexual conflict over sperm transfer. During simultaneous encounters with females males directed significantly more copulation attempts at larger females. Contrary to expectations, however, there was no resultant increase in either insemination success or the number of sperm transferred to larger females. Despite the high attempted copulation rate in our study (mean: 1.23 per min) only 54% of focal females were inseminated. This rate is similar to that reported in other studies (75% insemination success after 30 min: Evans, Pierotti, & Pilastro, 2003; 52% insemination success after 24 h: Pilastro et al., 1997). This suggests that cryptic female mate choice partly determines whether and how many sperm are transferred during male copulation attempts. The lack of a relationship between attempted copulation rate and insemination success also highlights the need to be cautious of using proxies such as attempted copulation rate when inferring reproductive success.

Conclusions

Males directed significantly more copulation attempts towards relatively larger females when encountering females simultaneously rather than sequentially. This reflects a general, but nonsignificant, trend across all taxa (mean effect size $r = 0.353$ versus 0.433: Dougherty & Shuker, 2014). Previous studies in a few other species have, however, shown similar results, with males being significantly less choosy during no-choice trials than dichotomous choice trials (effect sizes in Dougherty & Shuker, 2014). However, other species show high levels of choosiness even when mates are encountered sequentially. Lower consistency across species in how males respond to variation in encounter rate when compared to how females respond suggests that there may be sex differences in how mating ecology (e.g. natural encounter rate, density or sex ratio) influences the costs of rejecting mates. Further experiments investigating how variation in encounter rate influences male mate choice in carefully targeted species (e.g. to cover a range of natural mate encounter rates) would be useful to understand better why simultaneous versus sequential choice (i.e. experimentally 'two-choice' versus 'no-choice' tests) affects male mating preferences in some species but not in others.

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Appendix 4

Maternal effects on offspring size and number in mosquitofish, *Gambusia holbrooki*

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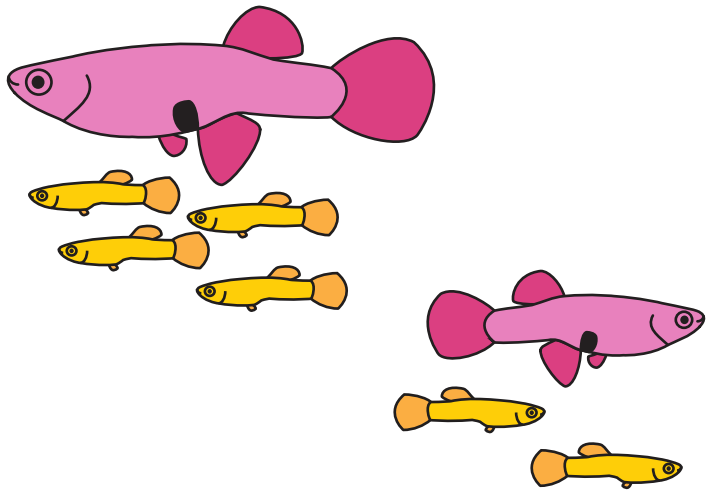

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Maternal effects on offspring size and number in mosquitofish, *Gambusia holbrooki*

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Maternal investment, nonlinear relationship, optimality model, trade-off.

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Abstract

Given a trade-off between offspring size and number, all mothers are predicted to produce the same optimal-sized offspring in a given environment. In many species, however, larger and/or older mothers produce bigger offspring. There are several hypotheses to explain this but they lack strong empirical support. In organisms with indeterminate growth, there is the additional problem that maternal size and age are positively correlated, so what are their relative roles in determining offspring size? To investigate this, we measured the natural relationship between maternal and offspring size in a wild population of *Gambusia holbrooki* (eastern mosquitofish), and experimentally disentangled the effects of maternal age and size on offspring size in the laboratory. In combination, our data indicate that the relationship between maternal and offspring size is nonlinear. Small mothers seem to produce larger than average offspring due to integer effects associated with very small broods. For extremely large mothers, which were only sampled in our wild data, these larger than average offspring may result from greater maternal resources or age effects. However, maternal age had no effect on offspring size or number in the laboratory experiment. Our results highlight the importance of sampling the full size–range of mothers when investigating maternal effects on offspring size. They also point to the difficulty of experimentally manipulating maternal size, because any change in size is invariably associated with a change in at least one factor affecting growth (be it temperature, food availability, or density) that might also have an indirect effect on offspring size.

Introduction

Maternal fitness depends on how many offspring are produced and how well these offspring survive and reproduce (i.e., their reproductive value). Mothers have finite resources to invest in reproduction so they face a trade-off between offspring size and fecundity (Roff 1983; Pollux and Reznick 2011). But what is the optimal offspring size? From a mother's perspective, larger offspring survive better than smaller ones (Einum and Fleming 1999; Johnston and Leggett 2002; Kuijper and Johnstone 2013; Omkar and Afaq 2013), but the size-fecundity trade-off counters an unfettered increase in offspring size (Trivers 1974). From the offspring's perspective, being as large as possible at birth is best (Blanckenhorn 2000; Rollinson and Hutchings 2013). It is, however, generally assumed that mothers control offspring size and have the upper hand in any parent–offspring conflict, especially when there is placental or maternal care (Steiger 2013). Most theoretical models therefore assume that offspring size maximizes maternal fitness (Marshall and Keough 2008).

The 1970s saw the development of a landmark model to determine the optimal maternal solution to the size-fecundity trade-off (Smith and Fretwell 1974). Smith and Fretwell modeled offspring fitness as a function with diminishing returns. That is, offspring fitness increases as mothers invest more, but the marginal rate of increase slows and approaches zero at the point where all resources are invested into one individual. The optimal offspring size occurs at the point of maximum returns on the offspring fitness curve. A shallower curve (i.e., smaller marginal gains) reflects a harsher environment, in which offspring need to be bigger to survive (Einum and Fleming 1999; Marshall et al. 2010). For example, the seed beetle *Stator limbatus* changes the size of its eggs to suit its host plant (Fox et al. 1997). Mothers produce larger eggs on plants that have lower larval survival, and therefore lay fewer eggs than when they lay eggs in a more benign environment.

Real-life patterns of offspring investment often defy the predictions of the optimality model for offspring size (Hutchings 1991; Marshall et al. 2010; Kindsvater et al. 2011). The model predicts that within a population, all mothers in the same environment should produce the same-sized offspring. Mothers with more resources should simply produce additional optimal-sized offspring. Maternal size is predicted to be positively correlated with offspring number, but uncorrelated with offspring size. A recent meta-analysis of 241 species from a wide range of taxa found, however, that maternal size tends to be positively correlated with both offspring number and size (Lim et al. 2014). While positive correlations between traits that are traded-off against one another can be an outcome of resource heterogeneity within a population (van Noordwijk and de Jong 1986), it is unclear why larger (resource-rich) mothers increase offspring size rather than offspring number. Furthermore, it has also been noted that older mothers produce larger offspring (Ribi and Gebhardt 1986; Glazier 1992; Ito 1997; Berkeley et al. 2004). Such maternal age effects could be frequently overlooked and attributed to maternal size due to a positive size–age correlation in many taxa (i.e., those with indeterminate growth) (Marshall et al. 2010).

There are several competing hypotheses to explain why maternal size and/or age affects (or is positively correlated with) offspring size (Marshall and Keough 2008). Most theoretical models focus on maternal size effects. For example, one of the earliest ideas was that the higher fecundity of larger mothers induces sibling competition, and that therefore their offspring need to be larger to compensate for this effect (Parker and Begon 1986). This explanation is more likely to apply in species when offspring do not disperse as juveniles (Kindsvater et al. 2012). A similar argument applies to a maternal age effect: life-history theory predicts that mothers face a trade-off between current and future reproduction (Williams 1966). If older mothers have a decreased likelihood of future reproduction (i.e., senescence), they are predicted to increase their investment in the current reproductive attempt (Pianka and Parker 1975). This may be accompanied by a concurrent increase in offspring size, to compensate for density-dependent sibling competition (Benton et al. 2008). Another model for a maternal age effect on offspring size asserts that if decreased reproductive effort increases longevity, then it is more advantageous for young mothers to reduce offspring size than number (assuming that it costs more to sacrifice fecundity; that is, lower fecundity has a stronger effect on fitness than does producing smaller sized offspring) (Kindsvater et al. 2012). In contrast, if older mothers have a lower expectation of future survival, they are predicted to produce the optimal offspring size irrespective of the associated survival risks.

Hypotheses for why larger or older mothers produce larger offspring generally lack robust corroborating empirical evidence (Marshall and Keough 2008). A key problem is identifying whether it is maternal age or size that is important, as these two factors are often correlated (Marshall et al. 2010). There are some studies in organisms with determinate growth that separate the effects of maternal age and size statistically (e.g., in the wandering albatross (*Diomedea exulans*) (Blanchard et al. 2007) and the wood duck (*Aix sponsa*) (Hepp and Kennamer 1993) maternal size, but not age, was correlated with offspring size). Experimental studies, however, are crucial to understand variation in life-history trade-offs, and how parent–offspring conflict over resource allocation into offspring size is resolved. In this study we use a species with indeterminate growth to experimentally tease apart maternal age and size to test their causal effects on offspring size.

Here, we investigate the effects of maternal age and size on offspring size and number in an organism with indeterminate growth, *Gambusia holbrooki* (eastern mosquitofish), a poeciliid fish with no postnatal parental care (Evans et al. 2011). This implies that mothers are under strong selection to produce optimal-sized offspring, because if they produce the "wrong-" sized offspring they cannot compensate by subsequently adjusting levels of care (Marshall et al. 2010; Steiger 2013). Sexually mature female *G. holbrooki* exhibit large size variation, ranging from 20 to 60 mm in standard length (SL) (Pyke 2005), which provides ample scope to study the effects of maternal size on offspring size. Their short life spans (generally <1 year in the wild) and brief breeding season in our study population (November to March) also mean that biologically significant age differences between *G. holbrooki* can readily be generated (Cabral and Marques 1999; Pérez-Bote and López 2005).

We investigate the relationship between maternal size/age, offspring size, and offspring number in a wild population of *G. holbrooki* and show that larger/older mothers have more and bigger offspring than smaller/younger mothers. We then experimentally manipulate the size and age of female fish in the laboratory to investigate the independent contributions of maternal size and age to this relationship. Our findings, and their interpretation, highlight the challenges associated with determining the factors causally responsible for variation in offspring size.

Materials and methods

Field methods

In January 2014, we captured 70 pregnant *G. holbrooki* from a pond in Canberra, Australia (35°18′27″ S°149°07′27.9″E). To identify pregnant *G. holbrooki*, we

indiscriminately caught fish with a hand net and deposited them into containers containing pond water. Pregnant females were identified as those with swollen abdomens. In the laboratory, we housed pregnant *G. holbrooki* individually in 1 L aquaria. Each tank contained a mesh divider, creating refugia for fry. We checked tanks for fry twice daily for 2 weeks after capture. Four females who did not give birth were discarded. We euthanized females after they had given birth and recorded their SL (SL = snout tip to base of caudal fin) (mm) by photographing them next to a scale ruler. We did not return fish to the wild because *G. holbrooki* are an invasive species in Australia (Macdonald et al. 2012) and it is illegal to do so. The size range of females that gave birth was 25.28–47.61 mm in length (n = 66; mean = 32.90; standard deviation [SD] = 6.10).

To measure the SL of fry, we took an overhead photograph of individual fry in water (5 mm deep) held in a small transparent container, placed atop 1 mm scale graph paper. The resultant images were analyzed using *Image J* (Schneider et al. 2012). Mean offspring size per female ranged from 6.68 to 7.82 mm (n = 66; mean = 7.22; SD = 0.29). We measured the SL of up to 10 fry per brood at birth (randomly selected from the tank they were born into), and noted the brood size. Brood size ranged from 1 to 104 (n = 66; mean = 23.74; SD = 19.93). We chose to measure a maximum of 10 fry per brood to strike a balance between obtaining an accurate estimate of the average offspring size within a brood, and obtaining comparable information on within-brood offspring size variation. Earlier pilot studies showed that there was very low variability in offspring size within a brood.

Experimental manipulation of maternal size and age

To disentangle the effects of maternal size and age on offspring size, we used the daughters of wild-caught *G. holbrooki* in laboratory breeding experiments. We had four cohorts of females: Large/Old (n = 56), Large/Young (n = 68), Small/Old (n = 72), and Small/Young (n = 84). In brief, we slowed the growth of the first, older cohort until the second, younger cohort caught up in size. We then split each cohort into two groups: One was placed in fast-growing conditions to become large and the other into slow-growing conditions to stay relatively small (Fig. 1). In the fast-growing conditions, we kept fish at low densities (initially 20 individuals per 90 L, reduced over time), at 28°C. In addition, fish were fed both *Artemia nauplii* and commercial fish flakes multiple times per day. Slow-growing conditions consisted of fish being kept at higher densities (eight individuals per 6.5 L), at a cooler temperature (19°C), where we fed them once daily, on a diet of *A. nauplii* (Vondracek et al. 1988; Pérez-Bote and López 2005). The range in length (in mm) of the females that gave birth was as follows: Large/Old: 33.00–37.82 (n = 23; mean = 35.40; SD = 1.24), Large/Young: 30.55–38.35 (n = 42; mean = 34.57; SD = 1.68), Small/Old: 23.77–29.52 (n = 36; mean = 26.78; SD = 1.40), Small/Young: 24.19–28.50 (n = 39; mean = 26.41; SD = 1.21).

Our laboratory-reared mothers were born in captivity in either November 2013 (Old) or January 2014 (Young). They were initially maintained at 28°C (five individuals per 2.5 L) and separated from males as soon as sexable (from 3 weeks of age onwards). To create similar sized

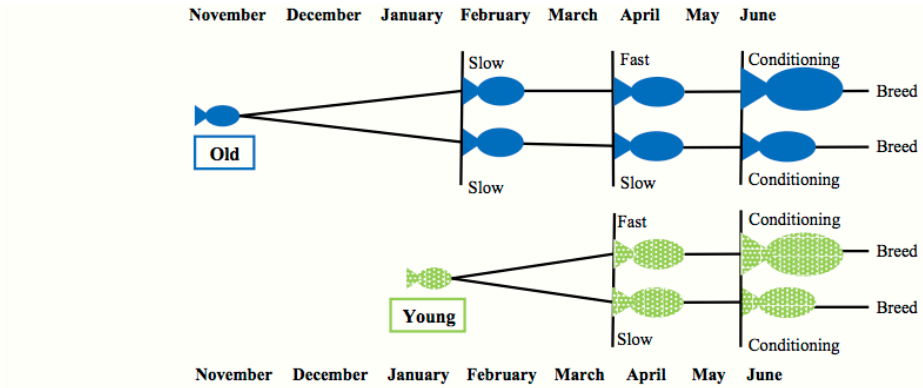

Figure 1. Experimental design to obtain four groups of females (Young/Old, Small/Large). Slow-growth conditions were as follows: 19°C, higher densities and fed once daily. Fast-growth conditions were as follows: 28°C, lower densities and fed ad libitum. All mothers were placed in a favorable ("conditioning") environment for 1 month prior to breeding.

individuals in the young and old-age classes, the Old females were kept in the slow-growing conditions for 2 months longer than the Young females (February to April, Fig. 1). This allowed the Young females to catch up in size to Old females by April (see Results).

In April 2014, we took half of the Old females, and half of the Young females, and housed them in fast-growing conditions, while the other half was housed in slow-growing conditions. To monitor the efficiency of our experimental treatments, we measured the size of a subsample of fish weekly. We marked 10 fish from each of the four groups (Large/Young, Large/Old, Small/Young, Small/Old) with fluorescent elastomer (Northwest Marine Technology, USA) injected subcutaneously behind the caudal fin.

To ensure females were in reproductive condition, they all underwent a conditioning period before breeding. They were kept at 28°C and fed a diet of *A. nauplii* and commercial fish flakes. The small treatment fish were, however, kept at higher densities than the large treatment fish (60 fish per 60 L compared to 10 fish per 90 L) to maintain the size difference during this time. The conditioning period lasted for 1 month, after which all females were reproductive (as indicated by two black spots near their genital opening; Pyke 2005). It is unavoidable that a mother's rearing conditions might affect offspring size due to her diet or rearing temperature rather than her size per se, but the conditioning period reduced the possibility of direct, short-term effects of maternal rearing conditions on offspring size. (It is obviously impossible to change a female's size and control for age without changing some aspect of her rearing conditions).

Breeding design

Females were set up to breed in June 2014 when the old and young cohorts were 7 and 5 months old, respectively. We placed one male with four females in 6.5-L aquaria. Males were first generation laboratory stock. After 1 week, we removed the male and separated the females into individual 1-L aquaria containing a mesh divider.

Three weeks after the male was first introduced (the minimum *G. holbrooki* gestation period; Pérez-Bote and López 2005), we began to check for fry twice daily. We measured the SL of the fry on the day they were born, and the SL of the mothers the following day, as per the protocol for wild-caught females. We discarded any females that had not produced offspring within 48 days of being with a male. A total of 142 of 280 females bred within the allotted period (the standard mean success rate in our laboratory). The breeding success of both Large and Small females was approximately equal (Large/Old: 41.1%, Large/Young: 61.8%, Small/Old: 51.4%, Small/Young: 47.6%), indicating that the conditioning period succeeded in controlling for the short-term condition of Large versus Small females.

All mothers and fry were given a unique ID before being photographed, to ensure that we made measurements blind to their treatment group. For analyses, we used the average size of offspring in a brood. Offspring size within broods was repeatable within each of the four treatment groups (intraclass correlations: Large/Old: $r = 0.50$, $n = 207$ fry, 23 mothers; Large/Young: $r = 0.55$, $n = 380$ fry, 42 mothers; Small/Old: $r = 0.59$, $n = 234$ fry, 36 mothers; Small/Young: $r = 0.85$, $n = 227$ fry, 39 mothers; all $P < 0.01$). There was no evidence that within-brood variation in offspring size differed as a result of maternal size or age (Size: $F_{1,130} = 0.08$, $P = 0.78$, Age: $F_{1,130} = 1.86$, $P = 0.18$; for analysis details see below). The range in length (mm) of the fry for the four groups of mothers was as follows: Large/Old: 6.84–7.58 ($n = 23$; mean = 7.30; SD = 0.21), Large/Young: 6.96–7.98 ($n = 42$; mean = 7.35; SD = 0.22), Small/Old: 6.87–7.95 ($n = 36$; mean = 7.42; SD = 0.27), and Small/Young: 6.51–8.84 ($n = 39$; mean = 7.48; SD = 0.49).

Before analysis, we removed two outliers as: (i) one brood consisted of a single offspring more than six standard deviations larger than the mean offspring size. It is highly likely that we overlooked its existence on its day of birth so that it was already at least 1 day old and (ii) one "Old/Small" mother was in the same size range as the "Old/Large" mothers, indicating that her response to the size manipulation treatment was atypical (including her in our analysis did not qualitatively alter our results).

Statistical analyses

To examine the effects of maternal size and age on offspring number and size, we performed a multivariate analysis of variance (MANOVA). We specified offspring size and number as dependent variables, and Age (Old/Young) and Size class (Large/Small) as fixed factors. We included the interaction term.

To examine the effects of maternal size and/or age on offspring size from wild-caught females, we performed partial correlation analyses to examine the relationships between maternal size, offspring size, and offspring number. We used the false discovery rate method to correct the *P*-values for multiple comparisons.

For direct comparison with wild-caught females, we also conducted bivariate correlations on the experimental females to examine the relationships between maternal size, offspring number and offspring size, pooling across maternal age and size classes.

Our laboratory experiment yielded different results to those for wild-caught females. To examine possible reasons for this, we conducted additional exploratory analy-

ses. First, to examine why the relationships between maternal and offspring size, as well as offspring number and offspring size, differed between experimental and wild-caught mothers, we repeated the original analyses on wild fish (partial correlations) but only included mothers within the size range generated in the laboratory (i.e., mothers <40 mm).

To investigate whether the relationship between offspring number and size differed between large and small mothers, we ran separate analysis of covariance (ANCOVA) for the experimental and wild-caught mothers, with offspring size as the dependent variable, maternal size and age (laboratory only) as fixed factors, and standardized offspring number (standardized so that mean = 0 and SD = 1; see Schielzeth 2010) as a covariate. We included the interaction term in the final model, as we were interested in any difference in the slope of the relationship. To generate comparable size classes for the wild-caught females, we classified them using the size ranges for small and large class laboratory-reared mothers (Small: 23.5–29.5 mm; Large: 30.5–40.0 mm).

To test whether the age or size treatments affected within-brood heterogeneity in offspring size, we ran an ANCOVA on offspring size variance for each brood, with age and size as fixed factors, and mean offspring size as a covariate.

For all models, we checked standardized residuals for normality. Where log-transformation of variables improved the normality of residuals we present these results. Effect sizes (Cohen's *d*) were calculated from partial eta squared values. We ran analyses with SPSS v. 22.0 (Armonk, NY, USA).

Results

Do larger mothers produce more and bigger offspring in the wild?

Larger wild-caught mothers had more and bigger offspring than smaller wild-caught mothers (offspring num-

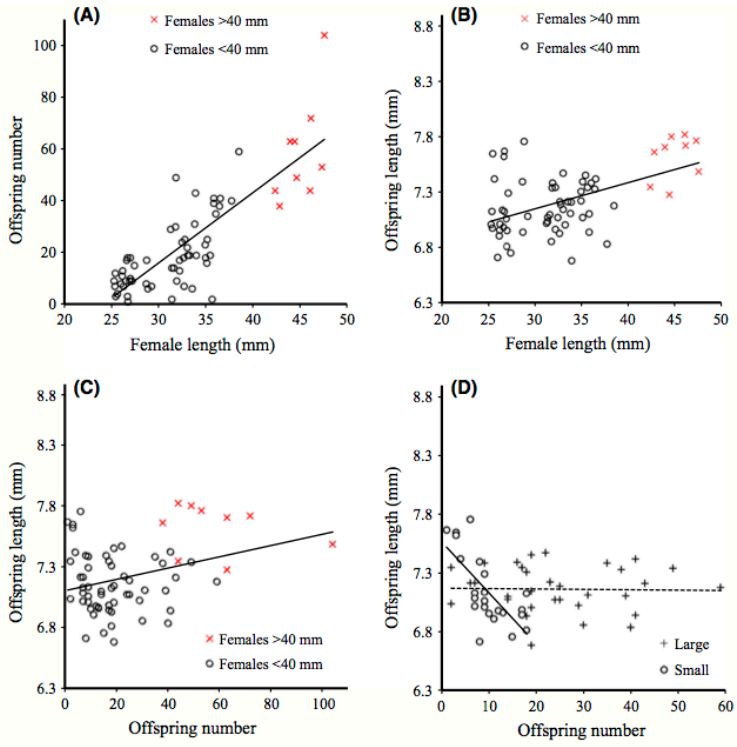

Figure 2. Wild population results. Analysis was on log-transformed data for panels B, C, and D. (A) The relationship between maternal size and number of offspring ($y = -65.48 + 2.71x$, $R^2 = 0.69$, $P < 0.001$); (B) the relationship between mother's size and offspring size ($y = 0.70 + 0.11x$, $R^2 = 0.22$, $P < 0.001$); (C) the relationship between the number of offspring in a brood and their average size ($y = 0.85 + 3.52{*}10^{-3}x$, $R^2 = 0.01$, $P = 0.50$); (D) the relationship between the number of offspring in a brood and their average size for mothers assigned to the Large and Small categories. There was a strong negative correlation between the number of offspring in a brood and their average size among small females. The regression lines are shown (Large: $y = 0.86 - 2.34{*}10^{-3}x$, $R^2 = 0.01$, $P = 0.71$; Small: $y = 0.90 - 0.05x$, $R^2 = 0.58$, $P < 0.001$).

ber: $r = 0.82$, 95% CI [0.72, 0.88], $n = 66$, $P < 0.001$, Fig. 2A; offspring size: $r = 0.59$, 95% CI [0.41, 0.73], $n = 66$, $P < 0.001$; Fig. 2B). Larger broods were comprised of smaller offspring ($r = -0.41$, 95% CI [−0.59, −0.19], $n = 66$, $P < 0.001$) (although with a standard bivariate correlation, which does not control for maternal size, there was no relationship between offspring number and offspring size for wild-caught mothers ($r = 0.08$, 95% CI [−0.16, 0.32], $n = 66$, $P = 0.502$; Fig. 2C).

How do maternal size and age affect the number and size of offspring?

Maternal size class, but not age class, affected offspring size (Table 1). However, in contrast to the pattern seen for wild-caught mothers, the larger laboratory-reared mothers actually produced smaller offspring ($r = -0.19$, n = 140, $P = 0.024$; Fig. 3B).

There was a significant interaction between the size class and age class of laboratory-reared mothers that affected brood size (Table 1). Larger mothers had significantly more offspring ($r = 0.68$, $n = 140$, $P < 0.001$; Fig. 4). For the large size class, old mothers produced significantly more offspring than young mothers, but there was no effect of age on fecundity for the small size class mothers (Fig. 4).

The effect of age class on offspring number might have been due to a small difference in the actual mean size of old and young mothers in the large size class (Fig. 5). To test for this, we restricted our analysis to large size-classes females and ran an ANCOVA with offspring number as a response variable, age class as a fixed factor, and standardized female SL (again, see Schielzeth 2010) as a covariate. Female size (SL) was now the only significant predictor of offspring number (Age class: $F_{1,\ 62} = 1.17$, $d = 0.28$, $P = 0.284$; Female SL: $F_{1,\ 62} = 19.13$, $d = 1.12$, $P < 0.001$; Age class*Female SL: $F_{1,\ 62} = 3.13$, $d = 0.45$, $P = 0.082$) despite the narrow range in female size (narrow because all females were in the same (i.e., large) size class).

Overall, there was a negative relationship between offspring number and size ($r = -0.57$, $n = 140$, $P < 0.001$); however, a significant interaction shows that this relationship was far stronger for small size class mothers than it was for large size class mothers (size class × offspring number interaction: $F_{1,133} = 3.97$, $d = 0.35$, $P = 0.048$) (Fig. 3D). In contrast, there was no difference in the size–number relationship between young and old-age class mothers ($F_{1,133} = 2.79$, $d = 0.29$, $P = 0.097$).

Table 1. Multivariate analysis of variance (MANOVA) test of between-subject results.

Source	Type III sum of squares	df	Mean square	*F*	*d*	*P*
Corrected model						
Offspring number	3436.26	3	1145.42	33.80	1.73	0.000
Offspring size	0.60	3	0.20	1.83	0.40	0.144
Intercept						
Offspring number	17721.77	1	17721.77	522.96	3.92	0.000
Offspring size	7236.54	1	7236.54	66479.16	44.22	0.000
Age						
Offspring number	225.55	1	225.55	6.66	0.44	0.011
Offspring size	0.12	1	0.12	1.08	0.18	0.300
Size						
Offspring number	3391.71	1	3391.71	100.09	1.72	**0.000**
Offspring size	0.51	1	0.51	4.67	0.37	**0.033**
Age*size						
Offspring number	140.88	1	140.88	4.16	0.35	**0.043**
Offspring size	0.00	1	0.00	0.00	0.01	0.944
Error						
Offspring number	4608.67	136	33.89			
Offspring size	14.80	136	0.11			
Total						
Number of offspring	24613.00	140				
Offspring size	7676.89	140				
Corrected total						
Number of offspring	8044.94	139				
Offspring size	15.40	139				

Age = age class (Old/Young); Size = Size Class (Large/Small).
Values in bold represent significant *P*-values.

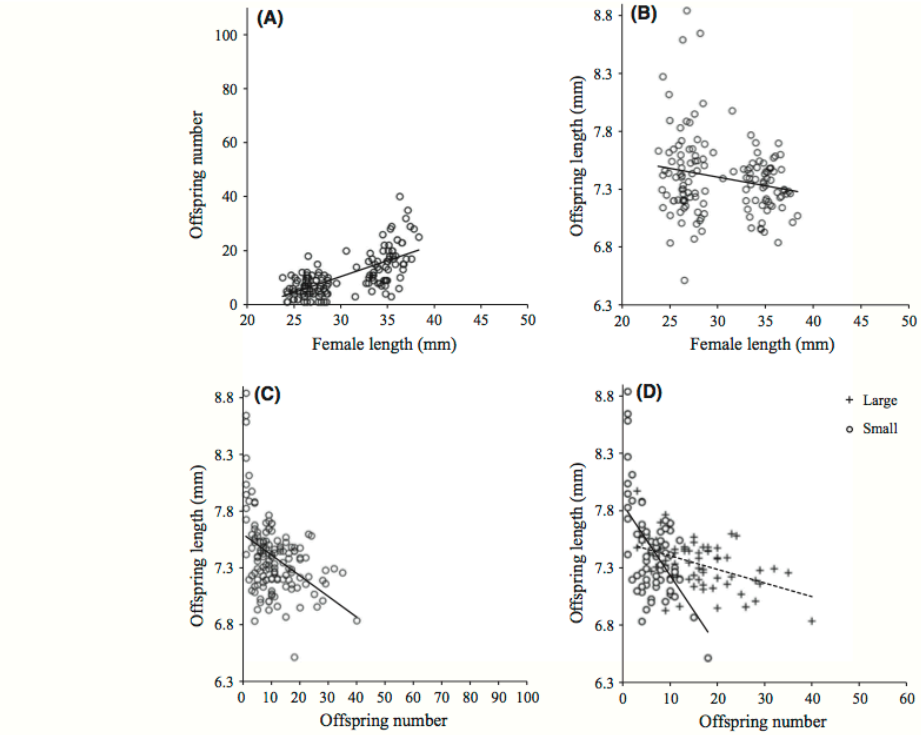

Figure 3. Experimental results for laboratory-reared fish. Analysis was on log-transformed data for panels B, C, and D. (A) The relationship between maternal size and number of offspring ($y = -24.96 + 1.18x$, $R^2 = 0.46$, $P < 0.001$); (B) the relationship between mother's size and offspring size ($y = 0.95 - 0.06x$, $R^2 = 0.06$, $P = 0.02$); (C) the relationship between the number of offspring in a brood and their average size ($y = 7.60 - 0.02x$, $R^2 = 0.17$, $P < 0001$); (D) there was a stronger negative correlation between the number of offspring in a brood and their average size among small females (Large: $y = 0.89 - 0.02x$, $R^2 = 0.19$, $P < 0.001$; Small: $y = 0.90 - 0.04x$, $R^2 = 0.42$, $P < 0.001$).

Why is there a difference between wild-caught mothers and laboratory-reared mothers?

The positive relationship between maternal and offspring size in wild-caught mothers was much weaker when we restricted the data to the maternal size range in our laboratory breeding experiment (SL <40 mm) ($r = 0.34$, 95% CI [0.09, 0.55], $n = 57$, $P = 0.01$). As before there was still a significant negative correlation between offspring number and size ($r = -0.44$, 95% CI [−0.63, −0.20], $n = 57$, $P = 0.021$), but this correlation remained even when female size was not controlled in the analysis ($r = -0.30$, 95% CI [−0.52, −0.05], $n = 57$, $P = 0.02$). When we categorized wild-caught females into the small and large size classes, it was clear that, as for the laboratory-reared mothers, smaller mothers had a steeper reduction in offspring size with increasing offspring number (size class*offspring number: $F_{1,\ 53} = 17.93$, $d = 1.15$, $P < 0.001$; Fig. 2D).

Discussion

Theory predicts that mothers should produce offspring of an optimal size and that mothers with more resources should produce more offspring rather than larger offspring (Smith and Fretwell 1974). This should create a positive relationship between maternal size and offspring number, but not between maternal size and offspring size. Our results indicate that these predictions hold for intermediate-sized mothers, but breakdown at extreme sizes. These findings highlight the importance of incorporating nonlinear relationships between life-history traits into predictions about optimal maternal allocation.

Relationship between maternal size/age and offspring size

We experimentally disentangled the effects of maternal size and age on offspring size in the laboratory. Maternal size, but not age, affected offspring size. Unexpectedly, however,

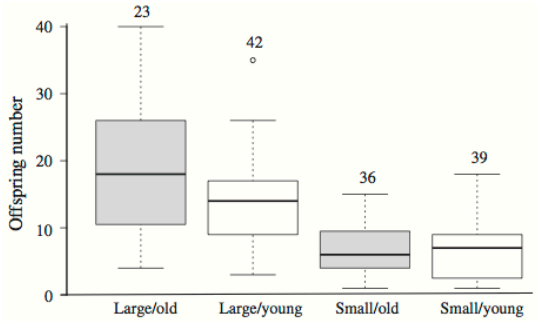

Figure 4. Box-and-whisker plot of the two-way interaction between female size class and female age class on offspring number. Open circles represent outliers. Sample sizes are displayed above the whiskers.

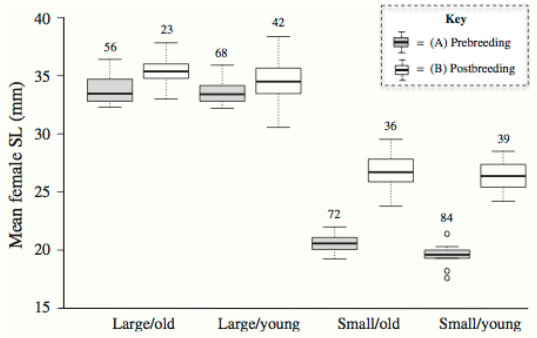

Figure 5. Female sizes measured (A) 7 days prior to being exposed to a male on 24th June 2014 (gray boxplots); (B) that eventually gave birth between 15th July and 11th August 2014 (white boxplots). The Small females continued to grow until they gave birth, reducing the ultimate size difference between the large and small size classes, and the Old females were slightly larger than the Young females. Open circles represent outliers. The number of females in each group is displayed above the whiskers. (Note that not all females bred, so this also affects the size differences between treatment groups for postbreeding females).

larger mothers produced smaller offspring. This was opposite to the relationship seen in the wild. Larger wild-caught mothers produced larger offspring, as seen in many other species (meta-analysis: Lim et al. 2014), including some poeciliid fish (Benejam et al. 2009; Swenton and Kodric-Brown 2012). Additional exploratory analyses revealed that the very largest wild *G. holbrooki* drove this trend. Wild-caught mothers in the size range of our laboratory-reared mothers (<40 mm SL) showed no relationship between maternal and offspring size. Our results suggest that the relationship between maternal and offspring size in *G. holbrooki* is nonlinear: Both small and large mothers have larger offspring than medium-sized mothers. This may explain the inconsistent results reported in the literature for the relationship between maternal and offspring size among *Gambusia*, where there is evidence of negative (Lim et al. 2014 – unpublished data cited in the meta-analysis digital repository), no (*Gambusia affinis*: Swenton and Kodric-Brown 2012), and positive correlations (*G. holbrooki*: Benejam et al. 2009; *Gambusia nobili*: Swenton and Kodric-Brown 2012). If the relationship between maternal and offspring size is nonlinear, then it is possible to obtain each of these results by sampling a subset of the full maternal size–range. For example, we would have found a negative relationship between maternal and offspring size if we had failed to sample very large females, a null relationship if we only sampled medium-sized females, and a positive relationship if we only sampled medium- and large-sized females.

The difference in the strength of the negative relationship between maternal and offspring size in laboratory-reared females of different size classes might be due to integer effects (and the fact that small females produce small broods). Because a mother's number of offspring must be an integer (they cannot produce a fraction of an offspring), the optimality model for offspring size fails at small brood sizes (Charnov and Downhower 1995; West et al. 2001). For example, if the total amount of resources a mother has to invest is 1.2 times the optimal level of investment per offspring, she can either produce two small or one large offspring (Charnov et al. 1995). If the fitness cost of producing offspring smaller than the optimal size is sufficiently high, then smaller broods will tend to have larger than average offspring. When brood size is plotted against offspring size, it is clear that the largest offspring occurred in the smallest broods (<5 offspring). We also note that our laboratory-reared *G. holbrooki* had smaller broods than the wild-caught females, making an integer effect less likely for the wild-caught females. To explore this idea, we re-analyzed the offspring size data after removing mothers who produced three or fewer offspring. Without those very small broods in the analysis, there is no interaction between offspring number and the size class of mothers affecting offspring size (ANCOVA: $F_{1,\ 123} = 0.09$, $P = 0.77$). This suggests that the stronger trade-off between offspring size and number exhibited by smaller females is driven by integer effects.

Relationship between maternal size/age and offspring number

Larger mothers had more offspring in both the wild and in laboratory-reared females. Greater fecundity among larger mothers is seen in most taxa (meta-analysis: Lim et al. 2014) and has been repeatedly demonstrated in poeciliid fish, including *G. holbrooki* (Edwards et al. 2006, 2010; Benejam et al. 2009). Furthermore, when we experi-

mentally disentangled the separate effects of maternal size and age we found no effect of age on fecundity. This result is contrary to the "cost of reproduction hypothesis" arising from life-history theory (Williams 1966; Skibiel et al. 2013). If senescence occurs then older mothers, who have a lower expectation of future reproductive success, are predicted to invest more in the present (e.g., terminal investment; Clutton-Brock 1984; Reznick 1985). In species such as *G. holbrooki* that show no postnatal parental care, this increased investment could only be mediated by an increase in offspring size and/or number. It follows from the optimality model for offspring size (Smith and Fretwell 1974) that increased investment should elevate fecundity. However, we did not see an age-mediated increase in offspring number, as reported in other species (Berteaux and Boutin 2000; Curtis Creighton et al. 2009), including *G. holbrooki* (Billman and Belk 2014). Possible explanations for the absence of an age effect are discussed below (see Study limitations).

Relationship between offspring number and offspring size

The largest mothers in the wild appeared to mask a trade-off between offspring number and size. An offspring size-fecundity trade-off must occur at the individual level because mothers only have finite resources to allocate toward offspring (Brown 2003). This relationship is often not detected at the population level because some mothers are "resource rich" and invest more in both traits (van Noordwijk and de Jong 1986). When maternal size was not accounted for our wild-caught fish showed no relationship between offspring number and size, as reported in two other studies on *Gambusia*: in feral Australian populations of *G. holbrooki* (Trendall 1982) and native American populations of *G. affinis* (Swenton and Kodric-Brown 2012). Once the largest mothers were removed from the analysis, however, offspring size and number were negatively correlated, as reported in many other poeciliid fish (Abney and Rakocinski 2004; Swenton and Kodric-Brown 2012). This change in the relationship between offspring size and number suggests that the largest mothers were able to invest more in both offspring size and number due to their greater resource status (van Noordwijk and de Jong 1986; Christians 2000), thereby obscuring the more general trade-off.

Study limitations

By experimentally generating large and small mothers of comparable age classes, we accumulated a number of confounding variables. The density, rearing temperature, and diet that our mothers experienced over the course of the experiment all differed between our treatment groups. Therefore, as is the case for wild *G. holbrooki*, the size and age of fish encompassed variation in the life history of the individuals. We cannot confirm that the apparent effects of maternal size were not due to an indirect effect of one of these confounding variables rather than a direct effect of maternal size. It is also possible that the slower growth environment experienced by older mothers may have masked an effect of age on fecundity. However, unless the effect of age exactly countered the effect of rearing environment, the fact that we observed no difference in offspring traits between old and young mothers of comparable size classes suggests that the additional 2 months that the older fish spent in the slow-growing conditions did not affect offspring traits. Another limitation of our study is that the age difference we generated between our old and young cohort (2 months) might have been insufficient to detect any effect of age on offspring traits. These limitations emphasize the difficulty of disentangling correlated variables: Independently manipulating age and size requires different rearing conditions, and if future studies seek to increase age difference they will also need to increase differences in rearing conditions to control for size. Future studies might do this using range of factors, each applied singly, so that they can identify whether it is maternal size per se or specific rearing conditions that generate offspring size differences.

Conclusion

We investigated the relationship between maternal size and offspring size in a wild population of *G. holbrooki*, and experimentally tested for effects of maternal size and age on offspring size and number. As predicted, maternal size was positively correlated with both offspring number and size in the wild, consistent with the general pattern seen in other species. This trend was, however, driven by very large mothers. The experimental results were unexpected. Larger mothers had higher fecundity, but smaller offspring, possibly due to integer effects arising in small broods. These effects seem to be independent of maternal age, at least in the laboratory. Unfortunately, it remains unclear whether the size or age of very large mothers drives the positive correlation between maternal and offspring size observed in the wild. Future experiments need to take into account the possibility that there are nonlinear relationships between life-history traits that influence maternal allocation toward offspring.

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Data Accessibility

Data deposited at Dryad: doi:10.5061/dryad.46r30.

Conflict of Interest

None declared.

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Appendix 5

Predictors of male insemination success in the mosquitofish (*Gambusia holbrooki*)

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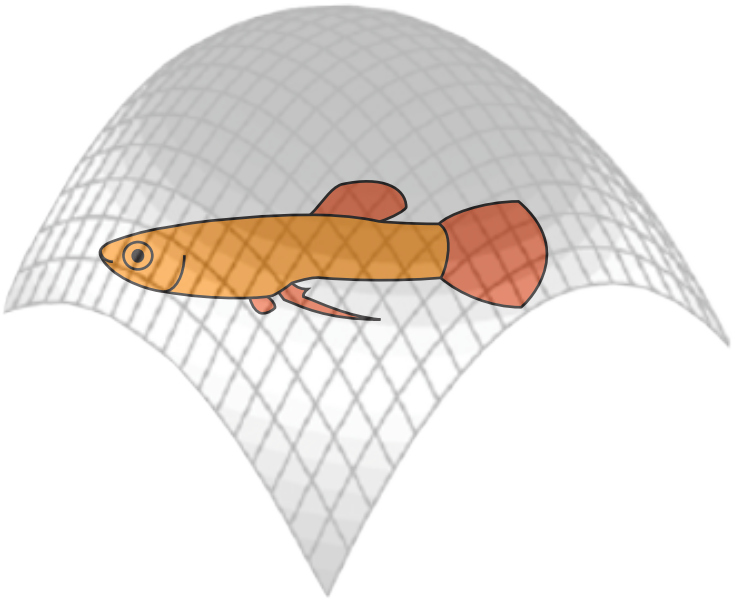

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Predictors of male insemination success in the mosquitofish (*Gambusia holbrooki*)

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Keywords
Correlational selection, insemination success, mate choice, mating success, poeciliid.

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Abstract

Identifying targets of selection is key to understanding the evolution of sexually selected behavioral and morphological traits. Many animals have coercive mating, yet little is known about whether and how mate choice operates when these are the dominant mating tactic. Here, we use multivariate selection analysis to examine the direction and shape of selection on male insemination success in the mosquitofish (*Gambusia holbrooki*). We found direct selection on only one of five measured traits, but correlational selection involving all five traits. Larger males with longer gonopodia and with intermediate sperm counts were more likely to inseminate females than smaller males with shorter gonopodia and extreme sperm counts. Our results highlight the need to investigate sexual selection using a multivariate framework even in species that lack complex sexual signals. Further, female choice appears to be important in driving the evolution of male sexual traits in this species where sexual coercion is the dominant mating tactic.

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Introduction

Studies of sexual selection generally focus on species in which males court females and have extravagant ornaments and/or complex courtship displays. Many researchers have adopted a multivariate approach to look at the resultant selection on male traits due to female mate choice (Lande and Arnold 1983; Blows and Brooks 2003). These studies have shown that selection due to female mate preferences is often both nonlinear (e.g., Greene et al. 2000; Brooks et al. 2005; Gerhardt and Brooks 2009) and favors specific trait combinations rather than acting independently on each trait (i.e., there is correlational selection) (e.g., Blows et al. 2003; LeBas et al. 2004; Bentsen et al. 2006; Ower et al. 2013). We therefore now have a good understanding of how sexual selection is mediated by females in such species.

Despite a focus on species with obvious female choice, in many other species sexual coercion is the dominant male mating tactic (reviewed in Cluttonbrock and Parker 1995; Chapman et al. 2003; Arnqvist and Rowe 2005). Males attempt to copulate through physical force and harassment (Cluttonbrock and Parker 1995), and it is expected that females either exert mate choice through mating resistance or mate indiscriminately to reduce the costs of sexual harassment (i.e., convenience polyandry: Thornhill and Alcock 1983) (Eberhard 2002). Well-known examples include premating struggles in waterstriders (Arnqvist 1992) and the many Poeciliid fishes where males incessantly harass females (Plath et al. 2007). These species rarely exhibit courtship displays or bear ornamental traits. This does not, however, preclude female-mediated sexual selection on males. For example, female mating resistance has the potential to generate variation in male mating success (Westneat et al. 1990; Wiley and Poston 1996; Jormalainen 1998; Gavrilets and Arnqvist 2001) due to sexual selection on traits that increase males' insemination success (e.g., genital shape in ground beetles, Yakami 2003; and bed bugs, Tadler 1999), and females might still actively bias male mating success by

preferentially associating with particular males (e.g., Japanese macaque, Soltis et al. 1997).

Relatively little is known about the targets or form of sexual selection on males in species with coercive mating systems, or the extent to which female mating preferences influence male reproductive success (Kokko 2005; Muller et al. 2011). Here, we investigate sexual selection on male eastern mosquitofish (*Gambusia holbrooki*), a species of poecillid fish in which males mate solely using a coercive tactic called "gonopodial thrusting". The gonopodium is a modified anal fin that acts as an intromittent organ. Males stealthily approach females from behind and then dart forward and attempt to insert the tip of the gonopodium into the female's genital opening (Langerhans 2011). We use standard multivariate selection analysis (Lande and Arnold 1983; Blows and Brooks 2003) to determine which male traits are correlated with insemination success, and the apparent direction and shape of selection on these traits. We focus our analysis on morphological and behavioral traits that have been shown to play important roles at various stages of mating. Further, we specifically explore insemination success in the absence of direct male–male competition to isolate effects of male–female interactions on male reproductive success.

Poecillid fish are known for their substantial variation in male body size and have become a model system for understanding how sexual selection drives such variation. It was originally assumed that female mate choice had little role in determining male mating success in *G. holbrooki* (Farr 1989), but later studies suggested that the probability of insemination is influenced by the amount of time since females have mated (Pilastro et al. 1997) and that females can influence the likelihood that forced copulation attempts result in actual genital contact under different environmental conditions (Condon and Wilson 2006). These findings suggest that females exert partial control over whether or not they mate. In addition, previous studies of *G. holbrooki* have shown that females prefer to associate with larger males (e.g., McPeek 1992; Bisazza et al. 2001; Kahn et al. 2012; but see: Bisazza and Marin 1991, 1995). Greater association time might increase mating success for large males if it increases access to females (Bisazza and Marin 1991; McPeek 1992). Larger males can also dominate their rivals for access to females (Bisazza and Marin 1991). However, in the absence of competitors, smaller males attempt more copulations than do larger males (Pilastro et al. 2003), and males that are relatively smaller than females have greater insemination success (Pilastro et al. 1997). Even so, it is still unclear whether this relationship is driven by female size, male size, or both (see fig. 1b of Pilastro et al. 1997). The seemingly higher mating success of small males has been suggested to result from their greater stealth and maneuverability (Bisazza and Marin 1995; Pilastro et al. 1997) but female size varies widely in *G. holbrooki* so the advantage of small male size could reflect the ability to sneak up on females who are *relatively* larger rather than effects due to *absolute* male size. The effects of gonopodium length on male mating success have received less attention, but studies of two *Gambusia* species indicate that females prefer longer gonopodia (*G. holbrooki*: Kahn et al. 2010; *G. affinis*: Langerhans et al. 2005). However, gonopodium length might also reduce male maneuverability: Male *G. affinis* with longer gonopodia relative to their body size have slower burst swimming speed (Langerhans et al. 2005). Here, we extend previous work by taking a multivariate approach to examining how male traits influence insemination success.

We predict that if male insemination success is primarily driven by male adeptness at coercion then smaller males will be more likely to inseminate females. Unlike previous studies that report a correlation between male size and insemination success (Pilastro et al. 1997), here we experimentally control female body size to isolate the effect of absolute male size. Alternatively, if insemination success is driven by female preferences, we predict that large males, and those with longer gonopodia will be more successful. If both processes operate, however, they might cancel each other out so that neither body nor gonopodium size have a detectable effect on insemination success.

Methods

Origin and maintenance of fish

Test females were offspring of wild-caught females collected in Canberra, Australia, in March 2013. Females were housed in single sex tanks (30–60 fish per 90 L) to ensure virginity. Males were collected from the wild in February 2014 and kept in the laboratory for 3–6 months prior to our experiment. All fish were maintained at 27°C on a 14:10 light:dark cycle and fed *Artemia salina* nauplii and commercial fish flakes twice daily. Test males were selected haphazardly from stock tanks to reflect the natural size distribution (standard length, SL range: 20.47–26.99 mm).

Experimental protocol

Prior to each experimental trial, males were stripped of sperm (details below) to fully deplete their sperm reserves then placed in a 7-L aquarium (17 × 28 × 15 cm) that was divided in two by a mesh barrier. On the other side of the barrier, we placed a stimulus female to prime sperm production (see Bozynski and Liley 2003). Stimulus

females varied in size, but our previous work showed that female size does not influence sperm priming in *G. holbrooki* (Head et al. 2015).

After 3 days, we stripped the male again to estimate his sperm count (3 days is enough time for males on our laboratory diet to replenish their sperm reserves (O'Dea et al. 2014)). After a further 3 days, we then replaced the stimulus female with a standard-sized female (350–450 g) and recorded the male's mating behavior. The sperm number on day 3 (i.e., after the first 3 days) is our best estimate of the likely amount of sperm a male had available for insemination at the start of his mating trial (i.e., based on replenishment 3 days after being stripped). Test females in mating trials were kept individually in 1-L tanks for 6 days prior to being introduced to a test male.

To begin a behavioral trial, we removed the mesh barrier and allowed the pair to interact. After two minutes, we began to record their behavior for 10 min. We recorded the time that a male spent following the female and how many mating attempts he directed toward her (see: Vega-Trejo et al. 2014). After another 20 min, both fish were removed from the tank, and we attempted to collect sperm from the test female's reproductive tract. In total, we ran 58 mating trials.

Collecting and counting sperm from males

Sperm were stripped from males following the methods of Matthews et al. (1997). Briefly, following anesthesia in an ice slurry, males were placed on their side on a glass slide under a dissecting microscope. The gonopodium was swung forward and pressure was gently applied to the abdomen to expel sperm. Using a 10-μL pipette, we transferred the stripped ejaculate to a microcentrifuge tube containing a known volume (100–300 μL) of saline solution (0.9% NaCl).

We counted sperm following the methods in Evans (Evans 2009). Briefly, samples were vortexed for one minute to break up sperm bundles and evenly distribute sperm throughout the sample. Then, 10 μL of the sample was placed on a Neubauer hemocytometer under ×400 magnification (Kiyowa, Medilux-12 microscope). We photographed five cells of the hemocytometer so that sperm could later be counted blind to treatment. The five counts were summed, and the total number of sperm per fish was calculated.

Collecting sperm from females

Within 10 min of the pair being separated, we anesthetized the female in an ice slurry and retrieved sperm, if present, from her gonoduct (see: Pilastro et al. 1997; Pilastro and Bisazza 1999). The female was placed ventral side up on a cradle under a dissecting microscope. A glass micropipette was then used to flush her gonoduct with 30 μL of saline solution (0.9% NaCl). We then vortexed the sample for 60 sec to break up sperm bundles and evenly distribute sperm throughout the sample. We placed 10 μL on a Neubauer hemocytometer for viewing under ×400 magnification (Kiyowa, Medilux-12 microscope). The presence or absence of sperm was recorded.

Data analysis

We used a standard multivariate selection analysis to estimate linear and nonlinear sexual selection on male phenotypes (Lande and Arnold 1983). We assigned males an absolute fitness score of 1 or 0 depending on whether or not sperm was extracted from his test female. This absolute fitness score was transformed to relative fitness by dividing by the mean fitness calculated across the experiment (Lande and Arnold 1983). We then fitted a linear regression model including five male phenotypic traits (body length, gonopodium length, estimated sperm number, time spent following the female, and number of mating attempts) as predictor variables and relative fitness as the response variable to estimate the vector of standardized linear selection gradients (β). All male traits were standardized (mean = 0; standard deviation = 1). A quadratic regression model including all the linear, quadratic, and cross-product terms was fitted to estimate the matrix of standardized nonlinear selection gradients (γ). To reflect actual selection, the quadratic regression coefficients were doubled (see: Stinchcombe et al. 2008).

Relative fitness was binomially distributed. This does not influence the sign or magnitude of selection gradients (Lande and Arnold 1983), but it presents problems with testing the significance of these gradients. Therefore, to assess the significance of our linear and nonlinear selection gradients, we used GLM with a quasibinomial error structure (Fairbairn and Preziosi 1996; Gershman et al. 2014). To test the overall contribution of linear and nonlinear effects in our models, we used partial *F*-tests (Chenoweth and Blows 2005). We also calculated phenotypic correlations between male traits. All statistical tests were run in R version 3.2.0 (R development core team 2012).

Results

Phenotypic correlations between male traits indicate that larger males had longer gonopodia, and that males that spent more time following females made more mating attempts (Table 1). There was also a nonsignificant trend for males with a longer gonopodia to have more sperm.

Table 1. Below the diagonal, the vector of standardized linear selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) for male phenotypic traits in *Gambusia holbrooki* (Significance was determined using GLM with a quasibinomial error structure). Above the diagonal (shaded) are the phenotypic correlations between traits. Estimates are followed by *P*-values in brackets.

		γ				
	β	Body length	Gonopodium length	Sperm number	Time following	Mating attempts
Body length	0.165 (0.324)	1.026 (0.073)	**0.664 (0.000)**	0.124 (0.356)	0.125 (0.349)	0.186 (0.162)
Gonopodium length	−0.043 (0.850)	−0.810 **(0.015)**	**1.398 (0.025)**	0.251 (0.057)	0.005 (0.970)	0.083 (0.534)
Sperm number	0.050 (0.797)	0.205 **(0.037)**	0.324 (0.928)	**−1.25 (0.022)**	−0.019 (0.886)	0.130 (0.332)
Time following	**−0.424 (0.038)**	−0.207 (0.577)	0.157 (0.287)	**−0.583 (0.044)**	0.240 (0.458)	**0.709 (0.000)**
Mating attempts	0.272 (0.121)	0.041 (0.810)	0.153 (0.527)	**0.422 (0.038)**	−0.065 (0.359)	0.094 (0.359)

Bold values are statistically significant.

In total, we retrieved sperm from 31 of the 58 test females. Although our relatively low sample size means that we have low statistical power our selection analysis using insemination success as the measure of fitness showed that, overall, linear selection did not significantly improve the fit of our model (partial F test: $F_{(5,52)} = 1.289$, $P = 0.283$), but that nonlinear selection did (partial F test: $F_{(15,37)} = 2.0297$, $P = 0.040$). Looking at individual male traits in our analysis showed that males that followed females for longer were significantly less likely to be successful (Table 1, Fig. 1A). There was also significant disruptive selection on male gonopodia length, and significant correlational selection due to an interaction between male body size and gonopodia length (Table 1). Large males with long gonopodia were more successful at inseminating females than were small males with short gonopodia (Fig. 1B).

There was stabilizing selection on the number of sperm, as well as correlational selection on sperm number due to interactions with the time spent following a female, the number of mating attempts, and body length. These selection gradients can be visualized in Figure 1. They show that males that produce intermediate amounts of sperm were relatively more successful at inseminating females if: (1) they had low rates of following (Fig. 1A); (2) made few mating attempts (Fig. 1C); (3) were large males (Fig. 1D).

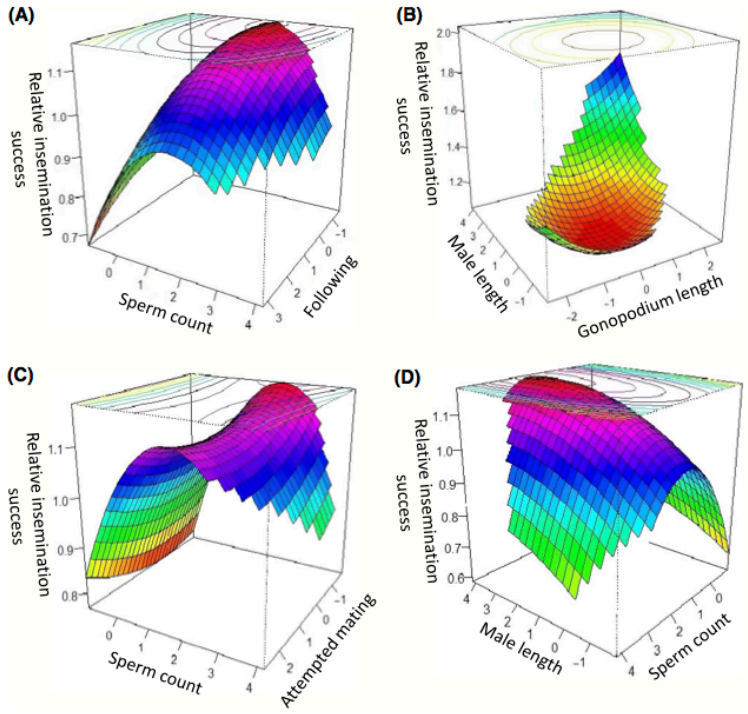

Figure 1. Response surfaces showing correlational selection. (A) the predicted relationship between sperm count, following and relative insemination success, (B) the predicted relationship between male length, gonopodium length, and relative insemination success, (C) the predicted relationship between sperm count, number of male mating attempts, and relative insemination success, (D) the predicted relationship between sperm count, male length, and relative insemination success. All phenotypic traits are standardized.

Discussion

Here, we examine selection on male traits predicted to affect male insemination success in *Gambusia holbrooki*, a species whose mating system is dominated by sexual coercion. We found no evidence for linear selection on four of the five traits we measured; however, there was both quadratic selection and correlational selection involving all five traits. Notably, large males with long gonopodia were significantly more likely to inseminate females than their counterparts. These results, combined with those of Kahn et al. (2010) showing that females prefer larger males and males with longer gonopodia, are consistent with females mediating male insemination success in *G. holbrooki*. Like studies of selection on complex sexual displays (e.g., Blows et al. 2003), our findings highlight the importance of examining multivariate selection on sexual traits.

Body size and gonopodium length

Male body size is a trait that often affects multiple mechanisms of sexual selection, a factor which might contribute to generally higher variation in male than female body size in many species (Wyman and Rowe 2014). In poeciliids, three mechanisms of sexual selection operate on male body size: male–male competition, sexual coercion, and female choice. Our finding that large males with long gonopodia were more likely than small males with short gonopodia to inseminate females suggests that insemination success in *G. holbrooki* is driven more by female mate preferences than a male's ability to force copulations. We did not directly tease apart the effects of sexual coercion and female mate choice on insemination success because these two mechanisms of selection occur simultaneously. However, the net selection for large males with large gonopodiums suggests that selection resulting from female choice overrides selection resulting from sexual coercion. We make this claim because Kahn et al. (2010) showed that females prefer to associate with large males that have longer gonopodia. This matches our own findings of which males were most successful. It is suggestive that female choice is still an important selective pressure in mating systems seemingly dominated by male sexual coercion (Eberhard 2002). Other studies that have teased apart the effects of sexual coercion and mate choice (e.g., Sih et al. 2002; Hall et al. 2008) also showed that female choice and sexual coercion act in opposing directions and that when mate choice occurs net selection on male traits is altered.

Previous studies have shown that large males dominate access to females when males compete directly (e.g., Bisazza and Marin 1995; Booksmythe et al. 2013). In our study, we deliberately excluded male–male interactions and found that larger males were more likely to inseminate females. Thus, sexual selection due to direct male–male competition and insemination success in the absence of rivals appear to act in concert, favoring larger males. This finding appears to be consistent across a variety of taxa (reviewed in Hunt et al. 2009). In contrast, however, Pilastro et al. (1997) found that a larger absolute difference in male and female size (i.e., relatively smaller males) increased the likelihood that a female was inseminated, suggesting that male–male competition and insemination ability create opposing sexual selection on male size. This difference between our study and that of Pilastro et al. (1997) could result from environmental differences that potentially influence the relative importance of selection arising due to sexual coercion and female choice (e.g., Sih et al. 2002). Testing for such environmental effects will be an interesting avenue for future research.

Behavior

Male following behavior was negatively related to insemination success. Although counterintuitive this relationship could arise if males pursue females less if they gain a successful insemination (e.g., a refractory period of decreased male sexual activity after mating has been shown in guppies (Pilastro and Bisazza 1999)). Our result indicates the potential for convenience polyandry (sensu Thornhill and Alcock 1983) and male sexual harassment to coevolve in *G. holbrooki*. Females could mate with males to reduce the level of harassment experienced, which could, in turn, select for increased harassment. The advantages of convenience polyandry depend, however, on the costs of mating such as an increased risk of contracting sexually transmitted diseases (STDs) (Lockhart et al. 1996), potential for injury (Crudgington and Siva-Jothy 2000; Blanckenhorn et al. 2002), or males transferring harmful substances in their ejaculates (e.g., Wigby and Chapman 2005). While there has been a lot of work on the costs of harassment for female poeciliids (including *Gambusia* spp (Plath et al. 2003)), the costs of mating are still poorly studied and deserve more attention. For instance, it would be interesting to test whether gonopodial intromission damages the female reproductive tract (Constantz 1984), or to test for sexually transmitted diseases, which are common in internally fertilizers (Lockhart et al. 1996).

Sperm count

We found nonlinear selection on male sperm number. Males that produced an intermediate amount of sperm after 3 days were significantly more likely to inseminate females than those producing higher or lower amounts. This was

unexpected. Why should males with large amounts of sperm have lower insemination success? One potential explanation is that sperm number is related to another unmeasured trait that also affects insemination success. This caveat about 'missing traits' is a limitation common to all selection analyses, as they are correlational and observed relationships between a trait and fitness are not necessarily causal (Ower et al. 2013). For example, a paternity study in guppies found negative directional selection on sperm production, which possibly represented a trade-off between pre- and postcopulatory traits under sexual selection (Head et al. 2008). Clearly, more work is needed to understand why, and how often, sperm number is not positively related to reproductive success in poecillid fishes.

Conclusions

Many of the male traits we measured were under correlational, but not directional, selection. This is unsurprising given that insemination success is determined by multiple mechanisms of sexual selection. The presence of correlational selection matters. It can have major consequences for the evolution of sexual traits and reproductive tactics. Correlational selection can drive the evolution of suites of integrated traits (Han and Brooks 2013), and build linkage disequilibrium between traits that are influenced by different genetic loci (Price and Langen 1992; Falconer and Mackay 1996; Lynch and Walsh 1998). Consequently, when selection drives a change in one trait, genetically correlated traits co-evolve (McGlothlin et al. 2005). Correlational selection could promote the evolution of alternative male reproductive tactics that are associated with certain male phenotypes leading, for example, to the evolution of small coercive males and large attractive males (seen in many species). Here, using an underutilized approach to remove direct male–male competition, we show that female choice could play an important role in driving the evolution of such reproductive tactics, even in species where ostensibly the only route to mating success is through male coercion.

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Conflict of Interest

None declared.

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